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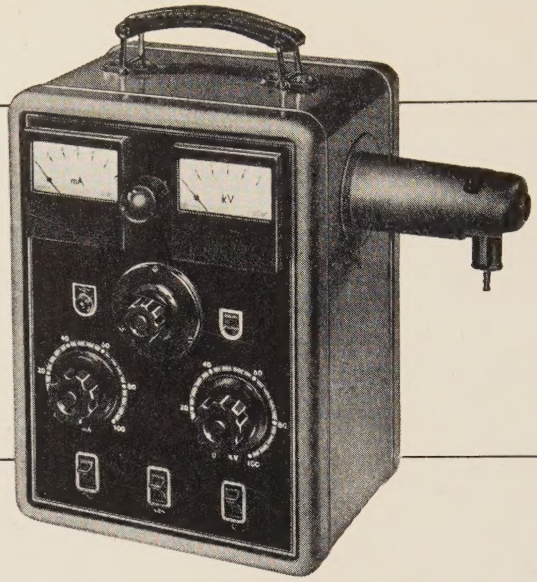

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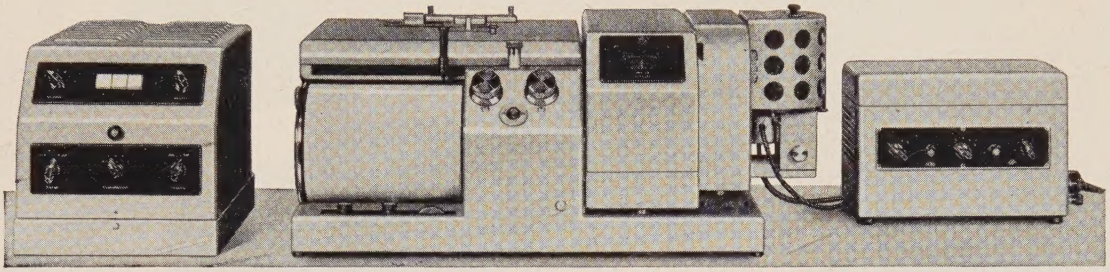

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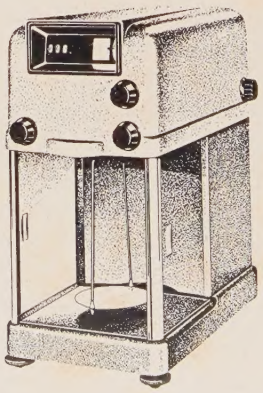

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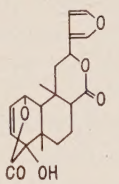
The Constitution of Limonin

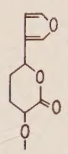
By D. ARIGONI[1], D. H. R. BARTON[2], E. J. COREY[3], and O. JEGER[1], in collaboration with L. CAGLIOTI[1], SUKH DEV[3], P. G. FERRINI[1], E. R. GLAZIER[3], A. MELERA[1], S. K. PRADHAN[2], K. SCHAFFNER[1], S. STERNHELL[2], J. F. TEMPLETON[2], and S. TOBINAGA[3]

The citrus bitter principle limonin was first isolated in 1841[4], but intensive investigation of its constitution did not commence until the last decade. The earlier literature has recently been summarised in detail[5] and we shall make reference here only to such of the earlier work as is especially pertinent to the derivation of the structure. The present communication represents a summary of the chemical evidence and conclusions to be reached from the work of three groups of investigators[1–3]. Experimental details of this work will be published separately in due course. Commencing in 1956 there has been in progress at the University of Glasgow a determination of the constitution and stereochemistry of limonin by the X-ray crystallographic method. This work, carried out under the leadership of Professor J. M. ROBERTSON, F.R.S., has been brought to a definitive conclusion and is summarised in the following communication. Professor ROBERTSON and his colleagues have at no time been informed of the progress of the chemical work until an exchange of letters dated September 11 1959 between one of us (D. H. R. B.) and the Glasgow crystallography group (see further below).

Before commencing an exposition of the chemical investigations it is essential to make a brief reference to what is known in the published literature about the constitution of limonin. Limonin, $C_{26}H_{30}O_8$ contains[5] two lactone rings, which can be opened reversibly, a β-substituted furan ring, a ketonic oxygen atom, and two ethereal oxygen rings. From infrared data both lactone rings are δ-constituted. Hydrogenation of limonin affords tetrahydrolimonin, $C_{26}H_{34}O_8$, and hexahydrolimoninic acid, $C_{26}H_{36}O_8$. These compounds both have the furan ring saturated and the acid is formed by hydrogenolysis of one of the lactone rings. Since tetrahydrolimonin is not an intermediate in the genesis of the acid one must conclude that the ether oxygen of the lactone that is cleaved is secured allylicly with respect to the furan ring. This is the same situation[6] as pertains in columbin (I), the main bitter principle of Colombo root. At first a relationship between limonin and columbin was suspected but, as shown in the sequel, limonin is a triterpenoid, not a diterpenoid, derivative and the supposed relationship is, in fact, misleading. Hexahydrolimoninic acid is an abnormally strong acid[7] and it is plausible to explain this by placing[5] an oxygen substituent α to the carboxylic acid group to give the part formula (II)[5] for limonin.



(I)



(II)

The ketone group of limonin has an infrared frequency which corresponds to that of a six (of higher) membered ketone. Reduction by the Ponndorff-Meerwein method affords[8] limonol, whereas borohydride reduction gives the epimeric epilimonol[5]. Since the latter compound is also formed by sodium amalgam reduction[5,9], a method which affords the more stable of a pair of stereoisomers, it can be assigned an equatorial orientation. Limonol, itself, must then be the axial isomer in agreement[10] with its mode of formation. We have converted[2] epilimonol into its chloro-acetate and thence into its iodoacetate. It is the latter compound which has been used for the X-ray crystallographic study of Professor J. M. ROBERTSON *et al.* The latter (see further below) confirm the equatorial character of the hydroxyl group in epilimonol.

[1] Organisch-chemisches Laboratorium, Eidg. Technische Hochschule, Zürich. This work was carried out with the financial support of the Schweiz. Nationalfonds zur Förderung der wissenschaftlichen Forschung.

[2] University of Glasgow and Imperial College, London.

[3] University of Illinois, Urbana; present address of E. J. C.: Harvard University, Cambridge (Mass.).

[4] BERNAYS, Liebigs Ann. *40*, 317 (1841).

[5] A. MELERA, K. SCHAFFNER, D. ARIGONI, and O. JEGER, Helv. chim. Acta *40*, 1420 (1957).

[6] D. H. R. BARTON and D. ELAD, J. chem. Soc. *1956*, 2085.

[7] O. H. EMERSON, J. Amer. chem. Soc. *74*, 688 (1952).

[8] B. V. CHANDLER and J. F. KEFFORD, Austr. J. Sci. *14*, 24 (1951).

[9] A. FUJITA and Y. HIROSE, J. pharm. Soc. Japan *74*, 365 (1954); *76*, 129 (1956).

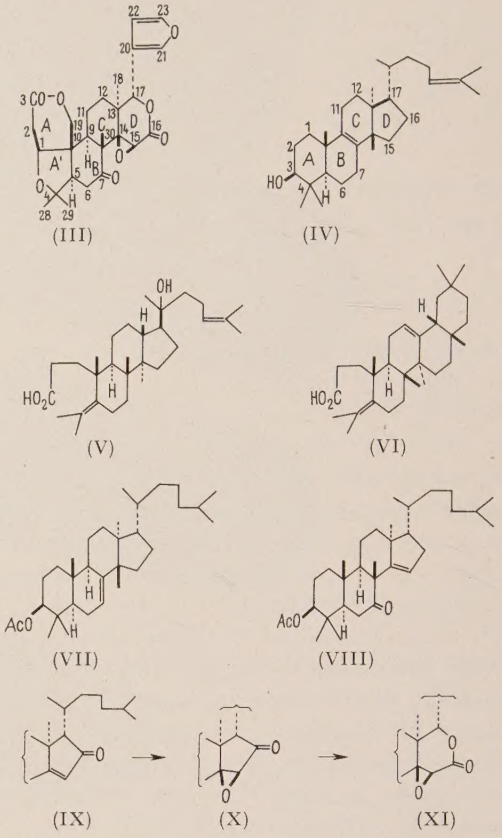
[10] D. H. R. BARTON, J. chem. Soc. *1953*, 1027.

Treatment of limonin with hydriodic acid[11] gives first desoxylimonin, $C_{26}H_{30}O_7$ and then citrolin, $C_{26}H_{28}O_6$. The former contains an $\alpha\beta$-unsaturated δ-lactone system[2,3] and has played an important role in the further elucidation of the constitution of limonin.

The molecular formula of limonin, when coupled with knowledge of the functional groups (see above), requires that the molecule be bicarbocyclic. Since drastic degradation of limonin[12,13] gives 1,2,5-trimethylnaphthalene one can make, with considerable reservation, the assumption that the two carbocyclic rings are both six-membered.

For the sake of clarity we must now state that the constitution and configuration of limonin is correctly represented as in (III). This constitution can be deduced, in main part, from the chemical work and is firmly established by the X-ray investigations. The stereochemistry is entirely the result of the X-ray work.

Biogenesis. – Limonin is revealed as a triterpenoid of the euphol (IV)[14] type from which four carbon atoms at the end of the side chain have been removed and carbons C_{20} to C_{23} then converted into a furan ring. A comparable process must be involved in the biogenesis of the cardiac aglycones. Ring A of the triterpenoid skeleton has been oxidatively cleaved between C_3 and C_4 and the C_3 carboxyl thus formed oxidatively cyclised onto C_{19}. There is excellent precedent for a C_3–C_4 biogenetic cleavage of the triterpenoid skeleton in the recently revealed structures of dammarenolic (V)[15] and nyctanthic (VI)[15,16] acids. The constitution of limonin also requires a Baeyer-Villiger oxidative cleavage of a ring D 16-ketone to give the ring D lactone. Finally one methyl group must be migrated from C_{14} to C_8 of the euphol skeleton. It is not without interest that chromic acid oxidation[17] of dihydrobutyrospermol acetate (VII) furnishes the 7-ketone (VIII). This type of compound could well be oxidised further to a 16-ketone, [as (IX)] epoxidised to the oxido-ketone [as (X)] and then subjected to Baeyer-Villiger oxidation to give a product (as XI) of the same partial structure as limonin. The numbering and lettering of the limonin formula (III) follow from these ideas as to its biogenesis. As would be expected the absolute configuration of tetrahydrolimonin is comparable with that expected for the ketone (VIII). Thus the rotatory dispersion curve of limonin[18] has a strong negative Cotton effect and corresponds in type to that of a saturated steroidal 7-ketone.



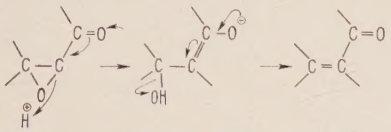
We now develop the detailed chemical evidence for the structure (III). For convenience we shall consider the molecule under sectional headings and recapitulate earlier work where appropriate and where it has been omitted from earlier reference above.

The Furan Ring.–The presence of a furan ring was first proposed by FUJITA and HIROSE[9]. Chemical confirmation of a β-substituted furan has been given by KUBOTA and TOKOROYAMA[19] and follows also from the following evidence. Treatment of limonol (XII) with alkali[1,3,5] gives a compound, $C_{21}H_{28}O_6$, designated[5] merolimonol (see further below). The loss of five carbons corresponds to the production of furan-3-aldehyde (XIII). This compound has now been isolated[3] and characterised as its 2,4-dinitrophenylhydrazone and by oxidation to 3-furoic acid. Finally, the nuclear magnetic resonance spectrum of limonin shows that it is a mono-β-substituted furan[20].

The Nature of Lactone Ring D. – The formation of the $\alpha\beta$-unsaturated lactone, desoxylimonin (XIV),

[11] T. A. GEISSMAN and V. TULAGIN, J. org. Chem. *11*, 760 (1946).
[12] G. KOLLER and H. CZERNY, Mh. Chem. *67*, 248 (1936).
[13] L. BRACHVOGEL, Arch. Pharmaz. *285*, 57 (1952).
[14] D. H. R. BARTON, J. F. MCGHIE, M. K. PRADHAN, and S. A. KNIGHT, Chem. & Ind. *1954*, 1325; J. chem. Soc. *1955*, 876. – D. ARIGONI, R. VITERBO, M. DÜNNENBERGER, O. JEGER, and L. RUZICKA, Helv. chim. Acta *37*, 2306 (1954); and references there cited.
[15] D. ARIGONI, D. H. R. BARTON, R. BERNASCONI, C. DJERASSI, J. S. MILLS, and R. WOLFF, Proc. chem. Soc. *1959*, 306.
[16] G. WHITHAM, Proc. chem. Soc. *1959*, 271.
[17] W. LAWRIE, W. HAMILTON, F. S. SPRING, and H. S. WATSON, J. chem. Soc. *1956*, 3272.
[18] C. DJERASSI, Bull. Soc. chim. France *1957*, 741; and references there cited. The rotatory dispersion curve of tetrahydrolimonin was determined in MeOH at the Postgraduate Medical School, London University. We thank Dr. W. KLYNE cordially for making this measurement and for helpful discussion.
[19] T. KUBOTA and T. TOKOROYAMA, Chem. & Ind. *1957*, 1298.
[20] E. J. COREY, G. SLOMP, SUKH DEV, S. TOBINAGA, and E. R. GLAZIER, J. Amer. chem. Soc. *80*, 1204 (1958).

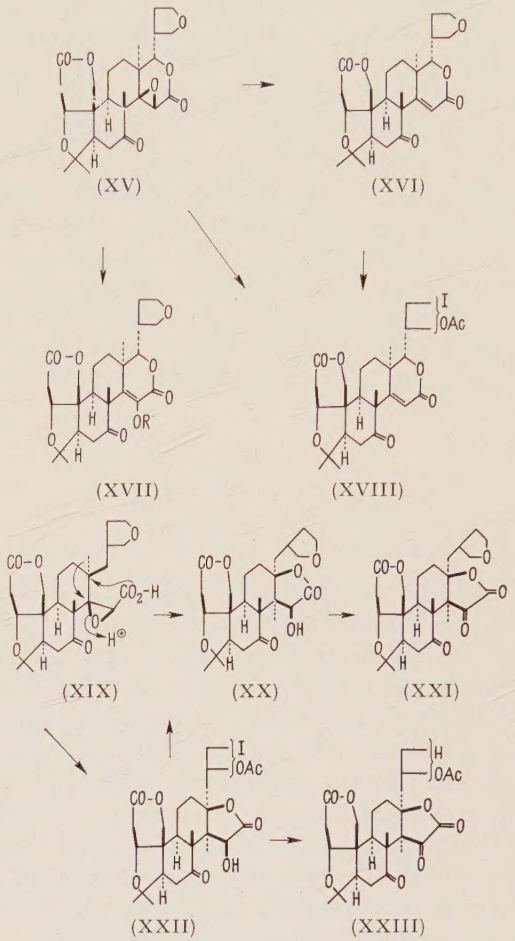
from the action of hydriodic acid on limonin suggests the possible presence of a 1,2-epoxide conjugated with the ring D lactone. Strong evidence for this grouping[3] is the fact that reduction of limonin with chromous chloride also gives desoxylimonin. Similarly tetrahydrolimonin (XV) furnishes desoxytetrahydrolimonin (XVI)[2,3]. Reductive elimination of 1,2-epoxides by chromous chloride is only possible if they are placed in conjugation with a carbonyl group[21] in accordance with the mechanism:



Furthermore decarboxyhexahydrolimoninic acid[22], the pyrolysis product of hexahydrolimoninic acid (XIX), contains an aldehyde group[1] (n. m. r. spectrum), as would be expected from the pyrolysis of a glycidic acid. Evidence that it is the D ring lactone which is involved and not the A ring lactone is given later. The presence of the glycidic lactone grouping in ring D is also shown by the following experiments[2]. Treatment of tetrahydrolimonin (XV) with hydrochloric-acetic acid mixture under controlled conditions gives the enol-lactone (XVII; R=H) characterised as its acetate (XVII; R=Ac). The ultraviolet absorption properties of these compounds show the presence of the enolised α-keto-lactone grouping and in confirmation[2] ozonolysis of (XVII; R=H), followed by mild hydrolysis, affords oxalic acid. Desoxytetrahydrolimonin itself is stable under the same conditions of acidity[2]. Treatment of desoxytetrahydrolimonin (XVI), or of tetrahydrolimonin (XV), with hydriodic acid in acetic acid-acetic anhydride at room temperature[2] served to open the tetrahydrofuran ring to give an iodo-acetate (XVIII). No other change in the molecule was produced (see further below).

When hexahydrolimoninic acid (XIX) was treated with acid[2] under comparable conditions to those described above it rearranged to a neutral isomer (XX) containing a new γ-lactone ring (infrared spectrum). Oxidation of this isomer with pyridine-chromium trioxide gave an α-keto-γ-lactone (XXI) in which the new ketone group could not be enolised. One must conclude, therefore, that the original isomerisation has placed a new carbon-carbon bond at the β-position in the lactone ring in agreement with the formulae given above. A comparable acid catalysed isomerisation of the ketone (VIII) has already been reported[17].

When hexahydrolimoninic acid (XIX) was treated with hydriodic acid[2] under the conditions applied above to desoxytetrahydrolimonin it furnished an analogous compound (XXII) with the tetrahydrofuran ring opened. This gave a desiodo-compound with zinc, whioh was oxidised to the α-keto-γ-lactone (XXIII) (infrared spectrum) with pyridine-chromium trioxide and gave (XX) on treatment with alkali.



The Environment of the Ketone Group in Ring B.— The environment of the ketone group in ring B was defined in an unexpectedly simple manner[2,23]. Limonin (III), dissolved in dry tert.-butanol containing potassium tert.-butoxide was rapidly autoxidised with uptake of one mole of oxygen. The highly crystalline acidic product (XXIV; *R*=H) was characterised as its acetate (XXIV; R=Ac). The presence of the diosphenol chromophore was shown by the spectroscopic properties of these compounds as well as by the further degradation of the chromophore described later. Analogous diosphenols, (XXV) and (XXVI), were obtained in good yield from, respectively, desoxylimonin (XIV) and tetrahydrolimonin (XV). The environment of the ketone group, as disclosed by these experiments, is also in accord with deuteration experiments[2,3] under alkaline conditions at room temperature. Two atoms of deuterium are rapidly incorporated, a third more slowly.

[21] W. COLE and P. L. JULIAN, J. org. Chem. *19*, 131 (1954).

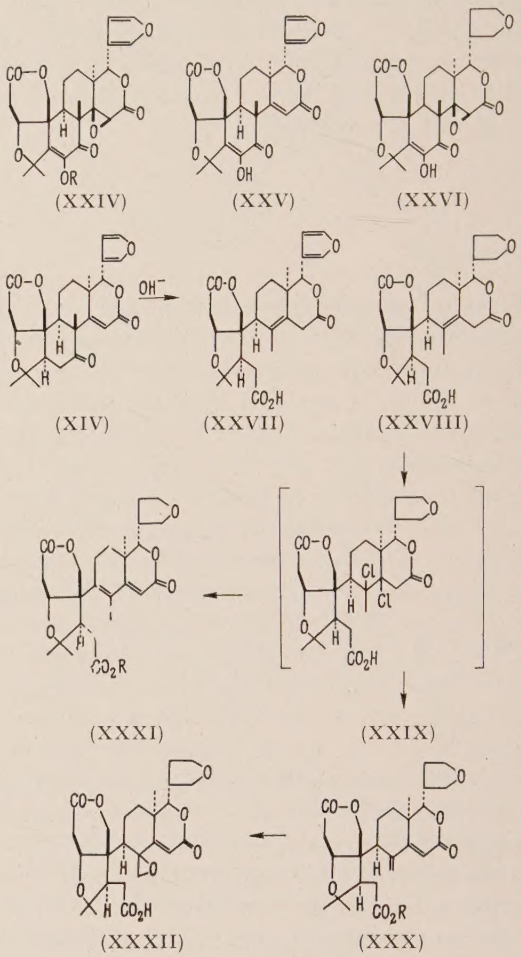
[22] R. S. ROSENFELD and K. HOFMANN, J. Amer. chem. Soc. *73*, 2491 (1951).

[23] The general scope of this reaction is currently being examined by two of us (D.H.R.B. and J.F.T.).

The Relationship of Ring B to Lactone Ring D. — Treatment of desoxylimonin (XIV) under mild alkaline conditions[2,3] causes cleavage of ring *B* to give a carboxylic acid (XXVII), conveniently designated desoxylimonic acid. Desoxylimonin oxime is stable[2] under the same conditions so that it is the carbonyl group of desoxylimonin that triggers the cleavage. Desoxylimonic acid, characterised as its methyl ester[2,3], showed only terminal absorption in the ultraviolet so that the conjugated δ-lactone ring of desoxylimonin has disappeared in the reaction. Similarly, desoxytetrahydrolimonin with aqueous alkali gives desoxytetrahydrolimonic acid (XXVIII). This acid is also obtainable from the iodo-acetate (XVIII) under the same conditions with reclosure of the tetrahydrofuran ring. The mechanism of this alkaline cleavage reaction, which does not, of course, occur with limonin, can be represented as follows:

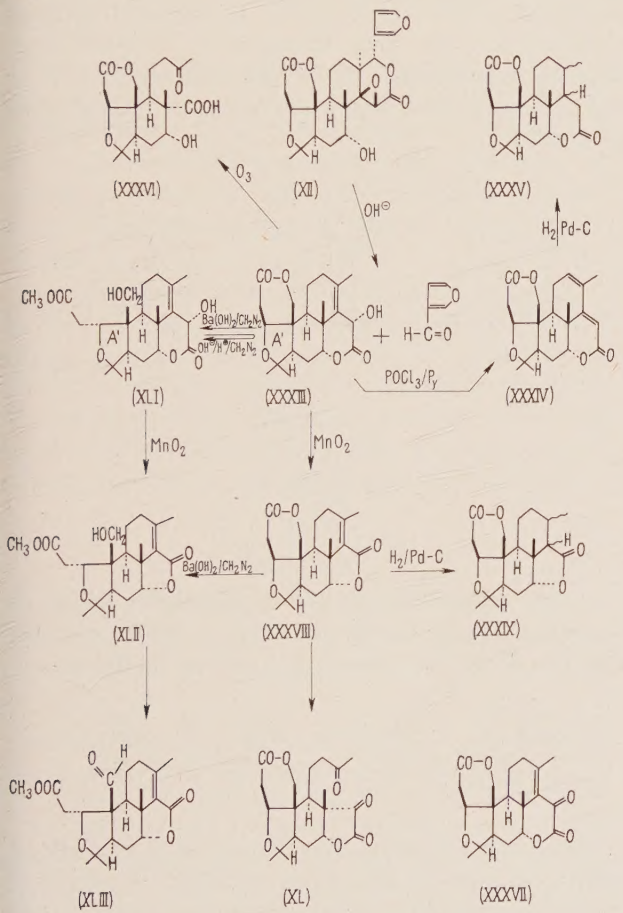
The further reactions of desoxytetrahydrolimonic acid have confirmed[2] the structure assigned. This acid reacts with one mole of chlorine to give an adduct (XXIX) (or equivalent) which is too unstable to be isolated. Heating *in vacuo* affords a mixture of two crystalline diene-acids. The major product has a broad absorption band with a maximum at 255 mμ and a low ε (7,800) in agreement with a *cisoid* diene as in (XXX; R=H). The minor product shows λ_{max} 230 and 284 mμ (ε=6,300 and 16,400 respectively) in agreement with the *transoid* diene formulation (XXXI; R=H). Both compounds were characterised as their methyl esters (XXX and XXXI; R=Me). The *cisoid* diene gives with per-acid an epoxide (XXXII) which has the normal spectrum of an $\alpha\beta$-unsaturated lactone. On ozonolysis (XXX; R=H) gives formaldehyde. This shows by chemical means the presence of a methyl group at C_8 [see (III)]. The formation of the *transoid* diene (XXXI) demonstrates also the presence of hydrogen at C_9.

Merolimonol and its Derivatives. — The relationship between the ketogroup of limonin and the lactone ring D indicated by these experiments can be proved independently from the chemistry of merolimonol[1,3,5], now assigned the constitution (XXXIII). Brief reference has been made to this compound before. As already reported it gives an acetate[5], now characterised[3] as a monoacetate, and is easily dehydrated to a doubly unsaturated conjugated lactone (XXXIV) which on hydrogenation affords a saturated tetrahydro-derivative (XXXV). We can now report[1] that ozonolysis of merolimonol gives a keto-acid (XXXVI) characterised as the methyl ester, the methyl ester acetate and as the methyl ester oxime. This compound is a methyl ketone since it affords iodoform with alkaline hypoiodite. Oxidation of merolimonol with manganese dioxide in chloroform[1] or in benzene[3] solution has given interesting results[1,3]. Besides the expected ketone[3] (XXXVII), there is formed[1,3] the



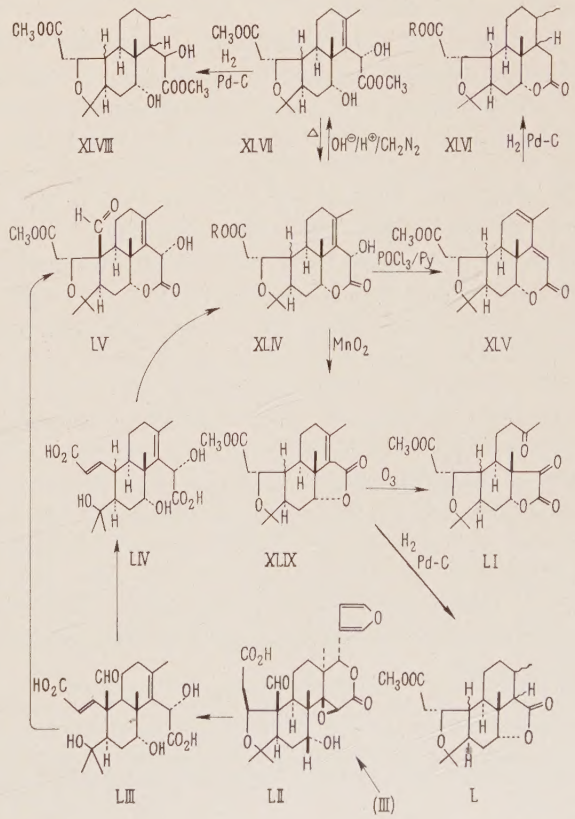
decarbonylated derivative (XXXVIII). The infrared and ultraviolet spectra of this compound show the presence of δ-lactone and $\alpha\beta$-unsaturated γ-lactone groupings. On hydrogenation it affords[1,3] a saturated dihydro-derivative (XXXIX) in which the γ-lactone could easily be seen in the infrared spectrum. This same compound was also obtained[3] by hydrogenating merolimonol to dihydromerolimonol followed by oxidation with pyridine-chromium trioxide. Decarbonylation is again involved[3] in this interesting reaction. Ozonolysis[1] or hydroxylation with osmium tetroxide followed by lead tetra-acetate cleavage[3] of the lactone (XXXVIII) affords an α-keto-lactone (XL) which is of importance for two reasons. First, the compound gives a positive iodoform test thus indicating a methyl ketone (see further under the discussion on n. m. r. spectra below); secondly, the α-keto-lactone does not enolise thus indicating (see also above) the presence of a substituent at C_8. As a whole, the results with merolimonol[1,3] characterise beyond question C_{12}, C_{13}, C_{14}, C_{15}, C_{16}, and C_{18} in their relationship with C_7 and C_8.

Some additional data pertaining to the chemistry of merolimonol can now be interpreted[1]. On treatment with barium hydroxide merolimonol gives an amorphous acid furnishing a crystalline methyl ester (XLI) with diazomethane. In this acid lactone ring A has been opened irreversibly in contrast to the behaviour of limonin and limonin derivatives in general. This can be simply explained by supposing that there is a reversible β-elimination and addition of the ethereal oxygen of ring A', thus permitting inversion at C_1 as already formulated in (XLI). Manganese dioxide oxidation of (XLI) affords[1] the expected γ-lactone (XLII) which on further oxidation with chromium trioxide gives the aldehyde (XLIII) (n. m. r. spectrum). This is important evidence that C_{19}, or other equivalent methyl carbon, is the terminus of lactone A (see further below). The mechanism which permits the merolimonol cleavage of limonol will be discussed in our[1,3] full papers.



The Chemistry of Limoclastic Acid.—When limonin is treated with alkali[5] under conditions significantly more vigorous than those required for the limonol → merolimonol change it affords, with a total loss of six carbon atoms, an acid, $C_{20}H_{28}O_6$. This substance is now assigned the structure (XLIV; R=H) and conveniently designated limoclastic acid[1]. Prior knowledge[5] of limoclastic acid can be summarised as follows. Its methyl ester is readily dehydrated by phosphorous oxychloride and pyridine to an anhydro-derivative (XLV) which has the same ultraviolet absorption spectrum as anhydromerolimonol (XXXIV). The anhydro-derivative, like (XXXIV), affords a satured tetrahydro-derivative (XLVI; R=Me) on hydrogenation which on alkaline hydrolysis furnishes the corresponding acid (XLVI; R=H). Methyl limoclastate is further characterised[5] as the monoacetate, giving a saturated dihydro-derivative on hydrogenation. Alkaline hydrolysis of methyl limoclastate (XLIV; R=Me) and esterification of the acidic product prior to relactonisation[1] affords a dimethyl ester (XLVII), characterised as a monoacetate, and giving back (XLIV; R=Me) on gentle pyrolysis. Hydrogenation of (XLVII) furnishes[1] a saturated dihydro-derivative (XLVIII) characterised as a diacetate. These experiments establish the functional groups of limoclastic acid and show that it is bicarbocyclic.

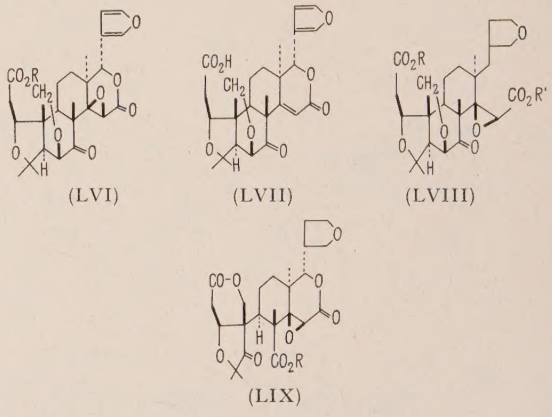
On oxidation with manganese dioxide methyl limoclastate (XLIV; R=Me) shows the same peculiar reactivity as merolimonol (see above) and affords a nor-compound (XLIX). On hydrogenation this gives a saturated dihydro-derivative (L) in which the γ-lactone grouping can be clearly discerned (infrared spectrum). Ozonolysis of (XLIX) also proceeds as with the merolimonol derivative (XXXVIII) and gives an α-keto-γ-lactone (LI) which does not enolise but does give the iodoform test in agreement with the presence of a methyl ketone grouping.



The stoichiometry of the limonin-limoclastic acid change requires that, if one fragment is furan-3-aldehyde (see above), a second fragment must be formic acid (or equivalent). Since the chemistry of limoclastic acid parallels exactly (see above) that of merolimonol we considered that the compounds were comparably formulated. Thus the ketone group of limonin must be converted by alkali induced intramolecular hydride transfer to a limonol type compound [as (LII)] which can now undergo the limonol-merolimonol change to furnish a compound [as (LIII)] which permits the deformylation process to occur by a conventional mechanism[24]. This gives in principle a structure [as (LIV)] which by re-addition of the tert.-hydroxyl to the $\alpha\beta$-unsaturated acid and lactonisation would afford limoclastic acid (XLIV; R=H). Interestingly enough a minor product from the alkaline degradation of limonin[1] is the aldehyde (LV). A direct connection between the limoclastic acid and merolimonol series of compounds has been secured as follows. The aldehyde (XLIII), to which reference has already been made above, is converted by the action of barium hydroxide followed by methylation of the product, into the lactone-ester (XLIX). Since (XLIII) is *stable under pyrolytic conditions*, the alkali must exert a special function in the decarbonylation reaction. The mechanism outlined above explains this in a unique manner and shows that ring A of limonin must be a δ-lactone with at least one α-hydrogen to permit reversible elimination of a β-placed ethereal oxygen. The terminus of the lactone ether-oxygen has already been established (see above).

Limonilic Acid and Derivatives. – Limonilic acid, $C_{26}H_{30}O_9$[7], was first obtained[11] by oxidation of limonin in alkaline solution with potassium manganate, but it is more readily available[7] in essentially quantitative yield by treatment of an alkaline solution of limonin with hypoiodite. Limonilic acid is now formulated as (LVI; R=H) on the basis of the following evidence. EMERSON[7] had shown that both tetrahydrolimonin (XV) and hexahydrolimoninic acid (XIX) gave limonilic acid type derivatives. The lactone ring modified in the limonilic acid reaction must therefore be lactone A and not lactone D (the hydrogenolysable lactone). In confirmation limonilic acid with either chromous chloride[3] or hydriodic acid[2] gives desoxylimonilic acid (LVII) in which the ring D lactone has been converted to an $\alpha\beta$-unsaturated lactone in the customary manner (see above). Furthermore[1] hydrogenation of limonilic acid followed by esterification affords the diester (LVIII; R=R'=Me) equally available from the hypoiodite oxidation[7] of hexahydrolimoninic acid and esterification.

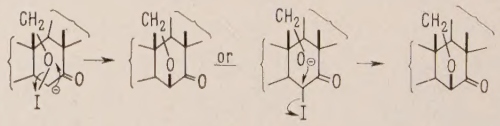
Contrary to earlier views[7] methyl limonilate[2] and the diester (LVIII; R=R'=Me)[1] have no hydroxyl group (infrared spectrum). With this fact established and, having regard to the stoichiometry of the reaction[7], limonilic acid must have either a second ketone or aldehyde function or else an ether grouping. Now the original ketone group of limonin, the presence of which is essential for a limonilic-type reaction, is profoundly modified in the limonilic acid reaction. It no longer exchanges readily with deuterium oxide in alkaline solution[3], no longer undergoes[2] the diosphenolation reaction (see above), has an ultraviolet maximum displaced to longer wavelengths and an infrared band at 1722 cm^{-1} displaced from its normal position near 1710 cm^{-1}. These facts are consistent with the presence of an axially oriented[25] ether substituent in the α-position with respect to the ketone. In agreement with this, reduction of limonilic acid with aluminium amalgam[1] gives back limonin. Furthermore[2], the degree of hindrance of the ketone group to carbonyl addition reagents has also been increased in limonilic acid, because its derivative, desoxylimonilic acid (LVII) no longer is subject to facile cleavage under mild alkaline conditions.



(LVI) (LVII) (LVIII)

(LIX)

On the basis of all this evidence limonilic acid must be formulated as already indicated in (LVI). The mechanism of its formation is presumably as follows:



The formation of an ether linkage is facilitated, no doubt, by a favourable steric situation. It will be of interest to see if such a process is of more general applicability.

Further Degradation of Ring B[2]. – The diosphenol (XXVI) from tetrahydrolimonin can be degraded smoothly by ozonolysis. This produces a crystalline nor-acid (LIX; R=H) further characterised as its

[24] Of course, the reaction conditions are alkaline so that anions are involved. This intricate process will be discussed in more detail elsewhere[1].

[25] R. C. COOKSON and S. H. DANDEGAONKER, J. chem. Soc. *1955*, 352. – G. BAUMGARTNER and C. TAMM, Helv. chim. Acta *38*, 441 (1955).

methyl ester (LIX; R=Me). When this nor-acid is treated with alkali it gives formaldehyde by a reversed aldol-type elimination. This proves that there is a $-CH_2-O-CO-$ group in limonin attached at either C_{10} or C_4.

The infrared spectrum of the methyl ester (LIX; R=Me) showed a band at 1760 cm^{-1}. This cannot be due to either lactones A or D and must be ascribed to the new ketone present in ring A'. This enhanced frequency for the ketone group is consistent with a cyclopentanone with an α-ethereal substituent such as is actually present.

Application of Nuclear Magnetic Resonance (n.m.r.) Spectroscopy. — Throughout this work one of the groups of investigators[3] has made extensive use of n. m. r. spectroscopy. In some cases this has suggested structures later confirmed by chemical methods and in others it has supplied valuable supporting evidence. A special section in this communication is clearly justified.

The n. m. r. signals which are characteristic of the 3-monosubstituted furan ring of limonin[20] are invariably absent from those derivatives lacking this unit (e.g. hexahydrolimoninic acid, merolimonol, methyl etiolimonate[9], and methyl limoclastate) and present in the remainder (e. g. citrolin, methyl limonilate).

At the other extreme of the n. m. r. spectrum lies the absorption due to the tertiary methyl groups which possess the most shielded protons. These signals are also extremely informative in the case of most limonin derivatives. With limonin ($CDCl_3$ solution) three sharp methyl peaks are observed at shift values (in parts per million from external methylene chloride as reference,

$$\frac{H - H_{CH_2Cl_2}}{H_{CH_2Cl_2}} \times 10^6$$

of + 4·16, + 4·29 and + 4·36 with a signal ratio of 1:2:1 and a total signal intensity of ca. 12 protons, a clear indication of the presence of four C-methyl groups.

In contrast, the n. m. r. spectrum of merolimonol shows absorption from only three methyl groups in this region. Three peaks are manifested at + 4·18, + 4·24, and + 4·35 which are cleanly separated with relative intensity 1:1:1 and total intensity of nine protons. Evidently one methyl group has been displaced in merolimonol and in agreement one finds a sharp band at + 3·4 (intensity ca. 3 protons) in the spectrum which corresponds to a methyl attached to a double bond. This conclusion is substantiated by the n. m. r. spectra of the γ, δ-dilactone XXXVIII (methyl peaks at + 3·08, + 4·07, + 4·20, and + 4·28), acetyl merolimonol and anhydromerolimonol (XXXIV). These data allow the assignment of an angular methyl substituent to C_{13} and are confirmed by the degradation of merolimonol to the diketo-dilactone (XL) which shows n. m. r. absorption characteristic of a methyl ketone at + 3·22 (acetone value + 3·25).

The n. m. r. spectrum of anhydromerolimonol (XXXIV) also exhibits a single peak at − 0·477 which is due to *two* olefinic protons. Since the diene system of XXXIV must possess a proton α to the carbonyl and a methyl at the γ-carbon, the remaining olefinic proton must be attached to the δ-carbon.

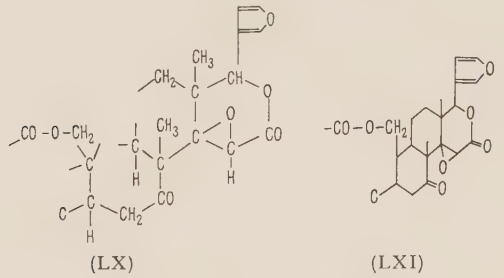
Strong evidence for the presence of an angular methyl substituent at C_8 came from the spectrum of methyl desoxylimonate (XXVII, methyl ester). The n. m. r. spectrum taken at 60 mc shows four sharp, cleanly resolved methyl peaks at + 3·67, + 4·07, + 4·17, and + 4·27 and no peaks due to olefinic protons (excluding the furan absorption). This shows definitely that the non-conjugated double bond in this substance is tetrasubstituted and that one of the attached groups is methyl (corresponding to the displaced peak at + 3·67). This methyl group can only be attached to C_8 since C_{14} substitution has been defined completely by the data pertaining to lactone ring D and merolimonol.

In this connection, it is also of interest that the n. m. r. spectra of desoxylimonin (XIV) and desoxytetrahydrolimonin show the attachment of only a single hydrogen to the olefinic linkage of ring D (sharp peak at − 1·42).

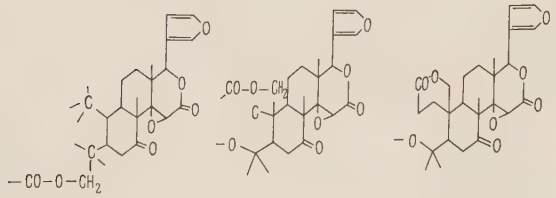
The Derivation of the Constitution of Limonin from Chemical Evidence. — The evidence outlined above provides conclusive proof for the presence of the system (LX) in limonin. If one has regard to the bicarbocyclic nature of the molecule and the formation of 1,2,5-trimethylnaphthalene on degradation (see above)[26] it is a possible assumption that two six-membered rings are present so that the rigidly derived partial structure can be expanded to (LXI) or (LXII). Structures like (LXI) and (LXII) immediately suggest a biogenesis from a triterpenoid (see above) and if one has regard to this and to the formation of acetone from the alkaline fusion of limonin and derivatives[7] then the constitution (LXI) can be expanded to (LXIII). Provided that there has been no reformation of carbon-carbon bonds in the biogenetic process we can then write (LXIV) as the constitution of limonin. Two formulae, (LXV) and (LXVI), derived from (LXIV) had been debated by us during the last six months. The origin of limoclastic acid, the properties of the nor-acid methyl ester (LIX; R=Me) (see above) and the course of attempts to degrade limonilic acid through its carboxyl group[2,3] tended to favour (LXVI) as against

[26] Dehydrogenation of merolimonol or of anhydrolimoclastic acid affords[5] a dimethylisopropylnaphthalene in which, from the ultraviolet absorption spectrum[1], the substituents are placed in positions 1, 2, and 6. From the constitution (III) of limonin this hydrocarbon should be 1,6-dimethyl-2-isopropylnaphthalene. Further work is in hand to confirm this[1].

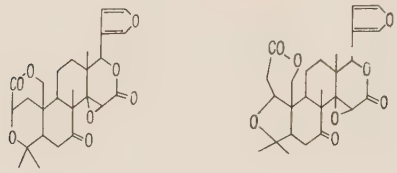
(LXV). The chemical evidence, however, still did not exclude some more exotic biogenesis of limonin, for example a condensation onto C_{11}, when the constitution from X-ray crystallographic work was announced by Professor M. Robertson in the exchange of letters already referred to above. At that time nothing was known from the chemical point of view as to the stereochemistry of limonin. The helpful collaboration of Professor M. Robertson *et al.* in making substantial contributions to our knowledge of limonin is gratefully appreciated by us.



(LX) (LXI)



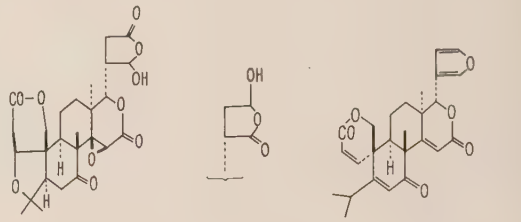
(LXII) (LXIII) (LXIV)



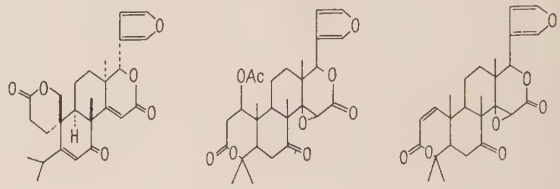
(LXV) (LXVI)

Other Aspects of Limonin Chemistry. — Limonexic acid[5,27], an oxidation product of limonin, must now be formulated as (LXVII) or (LXVIII). The relationship between this substance and limonin has already been established[5].

Citrolin, $C_{26}H_{28}O_6$, the product of vigorous hydriodic acid treatment of limonin (see above) can be obtained more conveniently by the action of hydrobromic acid[2] on desoxylimonin. Citrolin contains[7] the $\alpha\beta$-unsaturated lactone of desoxylimonin and has an $\alpha\beta$-unsaturated ketone grouping now to be placed in ring B. The most plausible formula for citrolin is (LXIX). In agreement the ultraviolet absorption curve of desoxylimonin, substrated from that of citrolin, gives a curve with maxima at 244 mμ ($\varepsilon = 14{,}000$) and 215 mμ ($\varepsilon = 8{,}000$), the latter possibly indicative of an additional $\alpha\beta$-unsaturated lactone grouping[2]. The infrared spectrum of citrolin is in much better agreement with the presence of two $\alpha\beta$-unsaturated δ-lactones than with one such lactone and one non-conjugated δ-lactone. Finally, selective catalytic hydrogenation of citrolin gives[2] a dihydro-derivative (LXX) shown by spectroscopic measurements to have one unsaturated and one saturated δ-lactone.



(LXVII) (LXVIII) (LXIX)



(LXX) (LXXI) (LXXII)

Some Related Bitter Principles. — It is probable that there are a number of bitter principles analogous in structure to limonin. We would mention especially nomilin[28], $C_{28}H_{34}O_9$, and the related obacunone[28,29], $C_{26}H_{30}O_7$. Recently[30] the functional groups of these compounds have been studied in some detail. It would appear that the formulae (LXXI) and (LXXII) are plausible and biogenetically attractive representations of nomilin and obacunone respectively.

Zusammenfassung

Limonin, ein Bitterstoff der Zusammensetzung $C_{26}H_{30}O_8$, kann aus verschiedenen Citrus-Arten isoliert werden. Frühere Untersuchungen haben gezeigt, dass der Naturstoff zwei carbocyclische Ringe, einen β-substituierten Furankern, zwei sechsgliedrige Lactone, ein Ketoncarbonyl und zwei intramolekulare Äthergruppierungen enthält. Neuere Abbauresultate, die in drei verschiedenen Laboratorien erzielt worden sind, erlauben, zusammen mit biogenetischen Betrachtungen, für diesen komplexen Naturstoff die Formel (LXVI) aufzustellen. Die gleiche Struktur, zusammen mit der noch unbekannten Stereochemie (vgl. III), ist ohne Kenntnis der chemischen Abbauresultate von J. M. Robertson *et al.* auf röntgenographischem Wege ermittelt worden. Auf Grund der Formel (III) kann Limonin als das Produkt eines weitgehenden oxydativen Abbaus des tetracyclischen Triterpens Euphol (IV) aufgefasst werden.

Wegweisend für die chemische Ableitung der Formel (LXVI) sind die Ergebnisse der alkalischen, sauren und oxydativen Behandlung des Limonins, welche einen Einblick in das reaktive Zusammenspiel der verschiedenen Sauerstoffunktionen gewähren. Die Natur und Umgebung des Lactonringes D folgt aus der Überführung von Limonin (III) in Desoxylimonin (XIV) sowie in eine Reihe von Produkten, in welchen der Epoxydring aufgespalten

[27] B. V. Chandler and J. F. Kefford, Austr. J. Sci. *13*, 112 (1951); *16*, 28 (1953).

[28] O. H. Emerson, J. Amer. chem. Soc. *70*, 545 (1948); *73*, 2621 (1951).

[29] F. Sondheimer, A. Meisels, and F. A. Kincl, J. org. Chem. *24*, 870 (1959).

[30] F. M. Dean and T. A. Geissman, J. org. Chem. *23*, 596 (1958).

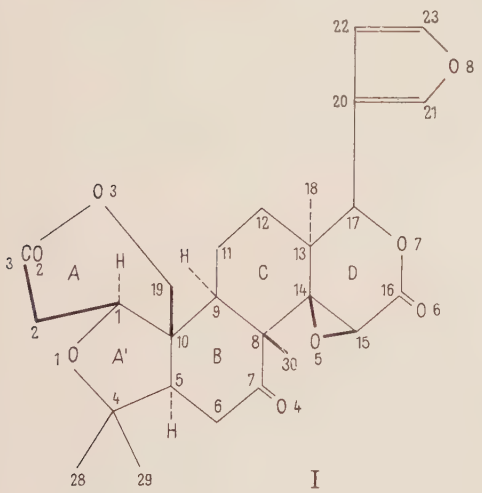
vorden ist. Die Umwandlung von Desoxylimonin (XIV) n Desoxylimonsäure (XXVII) gestattet, die Beziehung zwischen dem Lactonring D und dem Ketoncarbonyl festulegen. Zu gleichen Schlussfolgerungen führt auch die Untersuchung des Merolimonols (XXXIII), welches als Produkt des alkalischen Abbaus von Limonol (XII) anällt. Der oxydative Abbau von Ring B gibt Auskunft über die Substitution der C-Atome 5 und 6, während die Beziehung zwischen dem Ketoncarbonyl und dem Lactonring A durch die Bildung der Limonilsäure (LVI) festgelegt ist. Der drastische Abbau des Limonins mit Alkali führt zur Limoclastsäure (XLIV). Aus dieser Umwandlung ergibt sich die Natur des Lactonringes A sowie eine Haftstelle der verbleibenden Sauerstoffunktion. Die Integration der somit abgeleiteten Strukturelemente mit früheren Dehydrierungsversuchen erlaubt die für Limonin in Frage kommenden Strukturen auf wenige Möglichkeiten zu beschränken, unter welchen lediglich (LXVI) biogenetisch plausibel erscheint.

Anschliessend werden andere Aspekte der Chemie des Limonins und verwandter Bitterstoffe kurz erläutert.

The Structure of Limonin

By S. Arnott, A. W. Davie, J. M. Robertson, G. A. Sim, and D. G. Watson[1]

X-ray studies in this department have led to the elucidation of the crystal structure of *epi*-limonol iodoacetate and hence to the derivation of the molecular structure of limonin, $C_{26}H_{30}O_8$, the bitter principle of citrus fruits. Our results are summarised in formula I for limonin and in Figure 5 which shows the arrangement of the atoms in the crystal asymmetric unit.



I

The X-ray work commenced in 1956 and at that time our knowledge of the chemical structure was confined to an enumeration of the functional groups and the fact that two carbocyclic rings were necessary. There was insufficient basis on which to build any plausible trial structure, and it was clear that we could only hope to proceed by means of some phase determining heavy atom technique[2,3].

Professor Barton *et al.* supplied us with a variety of derivatives, and a preliminary study of these[4] indicated that the esters of *epi*-limonol were the most promising. The chloroacetate, which has two molecules in a monoclinic cell, space group $P2_1 - C_2^2$, was first studied. However, the presence of a screw axis introduces the ambiguity of a false symmetry centre between the two phase determining chlorine atoms, and work on this derivative was later abandoned in favour of the iodo-acetate of *epi*-limonol, $(C_{26}H_{31}O_8)COCH_2I$. This crystallises in the monoclinic system with cell dimensions $a = 15{\cdot}03$, $b = 12{\cdot}36$, $c = 15{\cdot}93$ Å, $\beta = 95° 12'$. There are four molecules in the unit cell, $D_m = 1{\cdot}426$, $D_x = 1{\cdot}441$ g·cm^{-3}, and the space group is again $P2_1 - C_2^2$.

In this space group the general positions are twofold, and hence, with four molecules in the unit cell, the asymmetric crystal unit consists of two chemical molecules. This is a formidable complication, because it means that the coordinates of 76 atoms other than hydrogen must be determined from the X-ray data. At a later stage of our analysis, however, this circumstance has been helpful. There is no symmetry relationship between the two molecules and the positions of all the atoms have been found and refined independently. The fact that these positions are now found to conform quite precisely to two chemically identical but differently oriented molecules is an important verification of the structure and the stereochemistry.

MoKα radiation was employed and the reflections were recorded photographically with Weissenberg and precession cameras from specimens measuring about $1{\cdot}0 \times 0{\cdot}2 \times 0{\cdot}02$ mm. Absorption corrections are small and were not applied. The extremely thin, flaky crystals made the collection of accurate data a very lengthy and tedious operation, but in the end 2927 structure factors were evaluated.

The positions of the iodine atoms were determined initially by means of two-dimensional and three-dimensional sharpened Patterson syntheses. A section of the three-dimensional Patterson function at $y = {}^1/_2$ is shown in Figure 1.

[1] Chemistry Department, The University, Glasgow (Scotland).
[2] J. M. Robertson, J. chem. Soc. *1935*, 615; *1936*, 1195.
[3] J. M. Robertson and I. Woodward, J. chem. Soc. *1937*, 219; *1940*, 36.
[4] S. Arnott and J. M. Robertson, Acta cryst. *12*, 75 (1959).

The iodine coordinates were obtained from the vector maps without ambiguity, and the fractional values, after refinement by later Fourier syntheses, are

	x	y	z
I(1)	0·1333	0·0367	0·3658
I(2)	0·0617	0·4633	0·9708

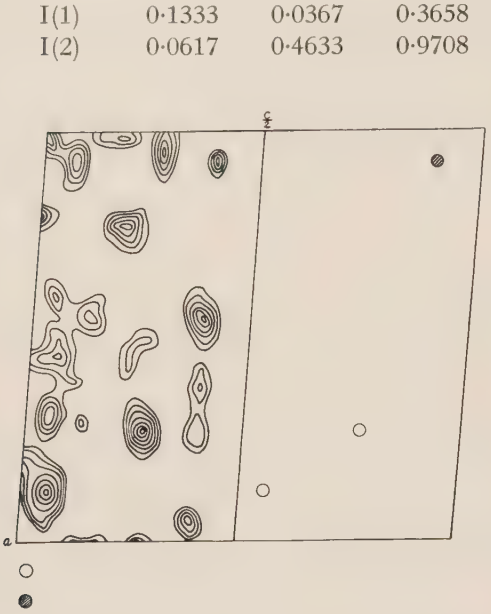


Fig. 1.—Harker section at $y = {}^1/_2$

With phase angles calculated from these coordinates (before refinement) various Fourier syntheses were now evaluated. Although it was not possible to interpret the two-dimensional projections obtained in this way, they led to a useful refinement of the iodine positions. The projection along the b axis has an effective centre of symmetry, and this is shown in Figure 2. The interpretation of this projection given in Figure 3 is based on our later results. It shows the complexity of the structure and the impossibility of attempting any definitive solution by these methods.

Fig. 2.—*Epi*-limonol iodoacetate. Electron density projection on (010)

In 1958 the first three-dimensional Fourier synthesis with phase angles based on the iodine atoms alone was evaluated and drawn out. Unfortunately a mistake in the programme for the electronic computer partially vitiated the results at this stage and much time was wasted in fruitless attempts at interpretation. When the error was detected, the synthesis was repeated. The coefficients of the Fourier series now employed were the observed structure amplitudes weighted according to the method proposed by Sim[5]. The adoption of this procedure permitted the inclusion at this early stage of all the structure-amplitude data rather than merely part of the data as is normally the case with other methods of assessing the reliability of the phase-angle information.

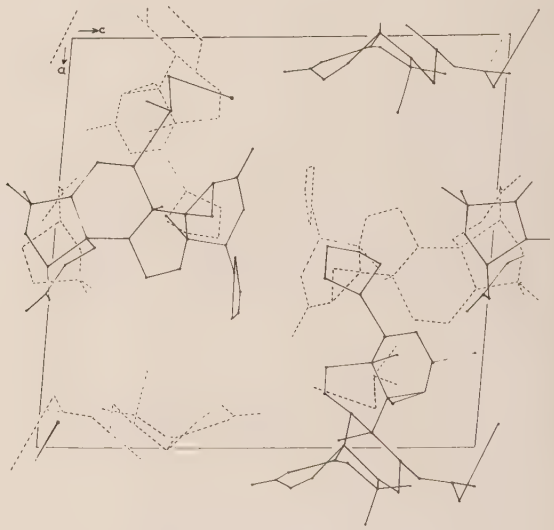
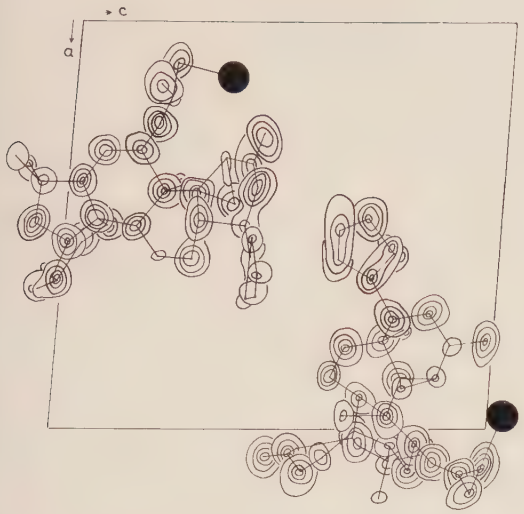


Fig. 3.—The final structure as projected on (010)

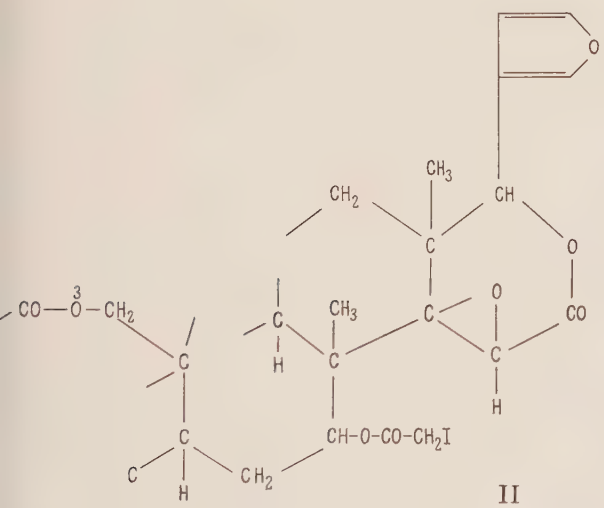
This approximate electron-density distribution was now compared with a function prepared from the three-dimensional Patterson synthesis by a superposition method[6,7], and a total of 50 atoms other than iodine were located with sufficient certainty to be included in the next structure-factor calculations. The phase angles obtained in these calculations were then used to derive an improved electron-density distribution in which all 76 atoms (C, O, and I) in the asymmetric unit could be placed. These atoms were now found to group themselves into two distinct and well separated molecules which were mutually consistent in the chemical sense. Part of this electron density distribution is shown by means of superimposed contour sections in Figure 4. The arrangement of the atoms in the asymmetric unit of the crystal structure is shown more clearly in Figure 5. This should be compared with Figure 3, which gives some idea of the mutual arrangement of the molecules in the unit cell.

[5] G. A. Sim, Acta cryst. *12*, 813 (1959).
[6] C. A. Beevers and J. H. Robertson, Acta cryst. *3*, 164 (1950).
[7] M. J. Buerger, Acta cryst. *4*, 531 (1951).



g. 4.—The third three-dimensional electron density distribution r *epi*-limonol iodoacetate shown by means of superimposed contour sections projected on (010).

At this stage it was not possible to distinguish be-ond all doubt between oxygen and carbon atoms. In articular in the lactone ring A it was not possible on ne basis of observed peak heights to rule out the possi-ility that O(3) might be carbon and C(2) oxygen. This lternative, however, is eliminated by the chemical vidence (see previous communication) which estab-shes unambiguously the partial structure II.



II

The approximate atomic positions are now being efined by least-squares and Fourier techniques. Two urther three-dimensional electron density distributions ave now been computed, with an increasing number f well defined atoms inserted in the phasing calcula-ons. The furan ring and carbon atom 17 of one mole-ule, which are distinctly resolved but still rather eak, have not yet been used in the structure factor calculations. The mean discrepancy between observed and calculated structure amplitudes is at present 22%. From the X-ray point of view a most important verification of the structure lies in the fact that the 76 independently resolved peaks are found to group themselves into two separate but chemically identical molecules.

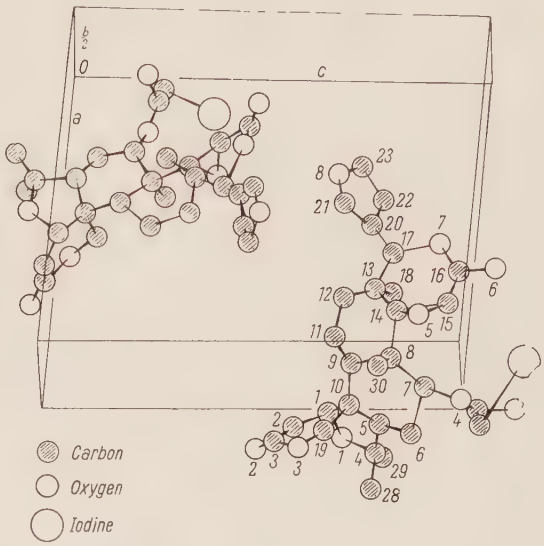


Fig. 5.—The arrangement of the atoms in the crystal asymmetric unit (two chemical molecules)

The stereochemistry of limonin is as indicated in formula I. In *epi*-limonol the hydroxyl group O(4) at C(7) is equatorial and the methyl group, C(30), at ring junction B/C is axial. Ring B has a chair and ring C a boat conformation. A full description with the coordinates of each atom will be given after further refinement has been completed. At the present stage of the work errors and uncertainties of a few tenths of an Angstrom may exist in some parts of the field.

We are grateful to Professor D. H. R. Barton for first suggesting this problem, and for making available supplies of the iodoacetate. Dr. O. S. Mills computed one electron-density distribution on the Manchester University Mercury computer. All the later calculations were carried out on the Glasgow University Deuce computer. Three of us (S. A., A. W. D., and D. G. W.) are also indebted to the Gourock Rope Work Company, the Department of Scientific and Industrial Research, and the Carnegie Trust for scholarships.

Zusammenfassung

Die Struktur des *epi*-Limonol-jodoacetates ($C_{26}H_{31}O_8$) $COCH_2I$, wurde durch dreidimensionale Röntgenanalyse bestimmt. Es gelang, die Lage sämtlicher Atome in zwei kristallographisch unabhängigen, jedoch chemisch identischen Molekeln zu ermitteln. Dieses Resultat stellt eine vollständige Bestimmung der Konstitution und der Stereochemie des Limonins dar.

Brèves communications - Kurze Mitteilungen
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Inhibition of Anaphylaxis in Mice by Pre-treatment with Cysteine, Lysine Ethyl Ester, and Tyrosine Ethyl Ester [1]

Immune hemolysis, according to the studies of Becker[2], proceeds by activation of the first component of complement (C^1 1) to form an esterase, with activity directed primarily toward *p*-toluene sulfonyl arginine methyl ester. This compound inhibits immune hemolysis, apparently by its action as a competitive substrate. Cushman *et al.*[3] also have demonstrated the inhibitory action of certain other compounds, among which were cysteine, lysine ethyl ester, and tyrosine ethyl ester. In view of increasing evidence for the participation of complement (C^1) as a primary factor in hypersensitivity reactions, this exploratory study on the effect of these materials on systemic anaphylaxis in the mouse was undertaken.

Methods. Swiss and CFW mice (6–8 weeks) obtained from Thomas Euers, Austin (Texas), were employed. Where mice of both sexes were used, they were distributed as uniformly as possible. Each experiment was performed with a separate lot of mice and was completed within one day. Antiserum was prepared in 3 adult New Zealand white rabbits by 3 weekly subcutaneous injections of 100 mg crystalline egg albumin (CEA) Armour) in complete Freund's adjuvant (Difco), with exsanguination at the fourth week. The pooled antiserum contained 390 μg antibody N/ml by the usual quantitative precipitin techniques. The following compounds were prepared at 0·1 M in buffered saline (0·0054 M Na_2HPO_4, 0·0013 M KH_2PO_4, 0·135 M NaCl) and, except where otherwise stated, were administered by intraperitoneal injection at a dosage of 1 ml/20 g body weight (0·005 M/kg): L-cysteine HCl, DL-tyrosine ethyl ester HCl (TEE), L-lysine ethyl ester diHCl (LEE), and *p*-toluene sulfonyl-L-arginine methyl ester HCl (TAME). All shocking injections were given intravenously into a tail vein. Rectal temperatures were recorded, just prior to the shocking injection and 30 min afterwards, using a direct reading thermistor device (Tele-Thermometer, Yellow Springs Inst. Co.). The rectal probe was introduced to 2 cm and the meter allowed to stabilize. This method has been found to be a reliable measure of the anaphylactic response in this laboratory and by others[4]. In this study anaphylaxis has been arbitrarily defined as reactions resulting in a 1°C drop 30 min after the shocking injection, or death within 30 min. This designation has the advantage of complete objectivity. Since most animals which show a 5°C drop within 30 min have lethal shocks, this value was assigned to animals dying within 30 min in calculating mean values. Additional information concerning methods will be given below.

Results. Preliminary toxicity studies with the 4 compounds showed TAME to be too toxic for further evaluation. Levels higher than 0·0015 M/kg uniformly resulted in deaths, while dosages less than this had no discernible effect on mouse anaphylaxis.

Reactions in actively sensitized mice (*Table I*). In Experiment 1 CFW mice were injected with 0·75 mg CEA in

[1] Aided by Research Grant (E 1563) from National Institute of Allergy and Infectious Diseases, USPHS.

[2] E. L. Becker, J. Immunol. *77*, 469 (1956).

[3] W. F. Cushman, E. L. Becker, and G. Wirtz, J. Immunol. *79*, 80 (1957).

[4] P. Morgan, N. P. Sherwood, and A. A. Werder, J. Immunol. *79*, 46 (1957).

Table I
Anaphylactic Reactions in Actively Sensitized Mice

Pre-Shock Treatment (IP Injection)	Pre-Shock Temperature (°C) Mean S. E.	Temperature 30 min after shock Mean* S. E.	Difference of Means	Deaths	Shock/ total	(% Shock)	P**
Exp. 1 ♀ CFW							
None	36·3 ± 0·3	30·9 ± 1·0	−5·4	6	10/10	(100)	—
Cysteine 60 min	35·0 ± 0·5	33·2 ± 1·1	−1·8	0	4/9	(44)	0·05
Lee 60 mm	36·9 ± 0·2	34·8 ± 1·2	−2·1	0	7/9	(78)	0·50
Saline 60 min	37·8 ± 0·1	34·6 ± 0·8	−3·2	0	4/5	(80)	0·75
Exp. 2 ♂ and ♀ Swiss							
None	36·4 ± 0·2	32·1 ± 0·3	−4·3	4	6/6	(100)	—
Cysteine 60 min	35·3 ± 0·3	33·8 ± 0·6	−1·5	0	3/6	(50)	0·20
Saline 60 min	36·7 ± 0·4	32·0 ± 0·5	−4·7	3	5/5	(100)	—
Exp. 3 ♂ Swiss							
None	37·6 ± 0·3	35·2 ± 0·6	−2·4	0	5/5	(100)	—
Cysteine 60 min	34·6 ± 0·3	33·6 ± 0·9	−1·0	1	1/5	(20)	0·10
Cystine 60 min	36·6 + 0·3	34·8 ± 1·0	−1·8	2	2/5	(40)	0·25
Lee 60 min	35·4 ± 0·4	34·8 ± 0·5	−0·6	0	2/5	(40)	0·25
Lysine 60 min	36·2 ± 0·1	32·6 ± 1·0	−3·6	4	5/5	(100)	—
Tee 60 min	34·4 ± 0·3	35·0 ± 0·1	+0·6	0	0/5	(0)	0·025
Tyrosine 60 min	35·0 ± 0·3	33·2 ± 1·4	−1·8	2	2/5	(40)	0·25

* Deaths considered as − 5·0°C Temperature drop.

** Determined from Chi-square values using Yates correction.

reund's adjuvant and on the 24th day given a shocking jection of 0·2 mg CEA in 0·2 ml. Experiments 2 and 3 ere similar except for the use of Swiss mice. Cysteine ven 1 h before shocking reduced the intensity and in-dence of the anaphylactic shock. LEE appeared to be ss effective, while TEE showed a significant inhibitory ffect. In most instances these compounds alone had a oderate hypothermic effect; however, the degree of ypothermia would not seem to be of sufficient magnitude o prevent an evaluation of the anaphylactic reactions y temperature depression. The absence of fatal shock in ne saline-treated animals in Experiment 1 should be oted. Statistical analysis of the combined Experiments 1, , and 3 showed cysteine treatment to be inhibitory at ne 98% level.

All surviving animals were injected again with the nocking dose of CEA 24 h after the initial injection. Both retreated and the untreated animals were equally refrac-ory to anaphylaxis. Animals which had been treated but ot shocked 24 h previously were not protected from hock.

Reactions in mice shocked by soluble antigen-antibody omplexes (*Table II*). In Experiment 4–8 a soluble antigen-ntibody complex prepared at 10 × optimal antigen con-entration (500 μg CEA N/ml antiserum) was employed. Antigen and Antiserum were allowed to react in buffered aline at 37°C for 30 min, then at 2°C for 48 h before entrifugation at 2°C. At this antigen level 40 μg ppt N vas formed. A total volume of 0·3 ml of the supernate btained was injected. These experiments demonstrated the variability of response in different groups of mice (e.g. compare cysteine at 60 min in Experiment 4 and 6). However, cysteine, LEE, and TEE were protective in most instances when administered 60 min prior to shocking. LEE produced the least hypothermia of the 3 compounds, while TEE gave the greatest temperature reductions. TEE was the most potent compound in modifying the anaphylactic response. In these, as in the previous experiments, surviving animals challenged 24 h after the initial injection were refractory to anaphylaxis. This could not be related to persistence of the drug effect.

Reactions in passively sensitized mice (*Table III*). All animals in Experiment 9 were shocked passively by the intravenous injection of 0·15 mg CEA in 0·2 ml within 1 min after the intravenous injection of 0·5 ml of a 1:5 dilution of antiserum (39 μg antibody N). Typical specific anaphylactic reactions are regularly produced in mice by this procedure[5]. Although statistical significance cannot be assigned to the findings in this small sample, results with cysteine and LEE at 60 min suggest a protective effect.

Discussion. A small measure of uncertainty in evaluating anaphylaxis by body temperature measurements has been introduced into these studies by a hypothermic response seen in some groups of animals after pretreatment with the compounds under study and before the shocking dose was given. No significant correlation was found to

[5] K. L. Burdon, Fed. Proc. *5*, 245 (1946).

Table II
Anaphylactic Reactions in Mice Using Soluble Antigen-Antibody Complex

Pre-Shock Treatment (IP Injection)	Pre-Shock temperature (°C) Mean S. E.	Temperature 30 min after shock Mean* S. E.	Difference of Means	Deaths	Shock/ total	(% Shock)	P**
Exp. 4 ♂ and ♀ *Swiss*							
None	37·2 ± 0·3	33·5 ± 0·5	−3·7	2	10/11	(91)	—
Cysteine 60 min	34·8 ± 0·3	34·6 ± 0·6	−0·2	1	6/14	(43)	0·10
Lee 60 min	36·4 ± 0·3	36·4 ± 0·4	0	0	3/10	(30)	0·025
Tee 60 min	35·7 ± 0·3	35·8 ± 0·4	+0·1	0	3/14	(21)	0·005
Saline 60 min	36·5 ± 0·4	33·2 ± 0·7	−3·3	1	12/15	(80)	0·50
Exp. 5 ♂ *and* ♀ *Swiss*							
None	37·0 ± 0·2	33·6 ± 0·5	−3·4	0	6/6	(100)	—
Cysteine 30 min	36·4 ± 0·3	35·0 ± 0·3	−1·4	0	3/4	(75)	0·70
Cysteine 40 min***	36·8 ± 0·3	35·5 ± 0·8	−1·3	0	3/6	(50)	0·20
Cysteine 60 min	34·4 ± 0·2	35·4 ± 0·5	+1·0	0	0/5	(0)	0·01
Lee 60 min	37·6 ± 0·5	36·8 ± 0·3	−0·8	0	3/5	(60)	0·80
Lee (0·05 *M*) 60 min	38·2 ± 0·2	36·2 ± 0·5	−2·0	0	5/5	(100)	—
Saline 60 min	37·4 ± 0·6	33·2 ± 1·5	−4·2	0	4/4	(100)	—
Exp. 6 ♂ *Swiss*							
None	37·4 ± 0·2	35·0 ± 0·7	−2·4	1	8/10	(80)	—
Cysteine 15 min	36·7 ± 0·1	34·3 ± 0·3	−2·4	1	13/15	(87)	0·50
Cysteine 30 min	35·7 ± 0·5	32·7 ± 0·6	−3·0	3	5/7	(71)	0·98
Cysteine 60 min	36·4 ± 0·2	34·7 ± 0·5	−1·7	2	9/16	(56)	0·50
Cysteine 150 min	37·0 ± 0·2	35·6 ± 0·5	−1·4	0	7/14	(50)	0·30
Cysteine 180 min	37·0 ± 0·3	35·7 ± 0·4	−1·3	0	5/7	(71)	0·98
Exp. 7 ♂ *and* ♀ *Swiss*							
None	36·5 ± 0·3	32·9 ± 0·4	−3·6	3	10/10	(100)	—
Tee 30 min	33·1 ± 0·3	33·1 ± 0·4	0	1	8/21	(38)	0·005
Tee 60 min	35·0 ± 0·3	34·2 ± 0·7	−0·8	0	4/9	(44)	0·05
Exp. 8 ♂ *Swiss*							
None	37·2 ± 0·2	33·6 ± 0·5	−3·6	2	18/19	(95)	—
Tee (0·1 *M*) 60 min	35·6 ± 0·4	33·7 ± 0·6	−1·9	2	9/14	(64)	0·10
Tee (0·2 *M*) 60 min	33·8 ± 0·2	33·2 ± 0·6	−0·6	3	4/19	(21)	0·001
Saline 60 min	37·8 ± 0·2	34·8 ± 0·9	−3·0	4	8/10	(80)	0·70

* Deaths considered as — 5·0°C temperature drop.
** Determined from Chi-square values using Yates correction.
*** Cysteine injected intravenously.

Table III
Anaphylaxis in Passively Sensitized Mice

Pre-Shock Treatment (IP Injection)	Pre-Shock Temperature (°C) Mean S. E.	Temperature 30 min after shock Mean* S. E.	Difference of Means	Deaths	Shock/ total	(% Shock)	P**
Exp. 9 ♂ *and* ♀ *Mice*							
None	36·4 ± 0·2	33·8 ± 0·8	−2·6	0	4/5	(80)	—
Cysteine 30 min.	35·0 ± 0·2	33·2 ± 0·6	−1·8	1	3/4	(75)	0·50
Cysteine 60 min.	35·0 ± 0·6	33·6 ± 1·2	−1·4	0	1/5	(20)	0·25
Cysteine 120 min.	35·0 + 0·5	34·2 ± 0·7	−0·8	0	2/5	(40)	0·70
Tee 30 min.	34·0 ± 0·6	32·0 ± 0·7	−2·0	1	4/5	(80)	—
Tee 60 min.	36·4 ± 0·5	35·4 ± 0·8	−1·0	0	3/5	(60)	—
Tee 120 min.	37·2 ± 0·2	34.0 ± 0·3	−3·2	0	5/5	(100)	—
Lee 30 min.	36·0 ± 0·3	33·6 ± 0·8	−2·4	0	3/5	(60)	—
Lee 60 min.	36·2 ± 0·5	36·5 ± 0·4	+0·3	0	0/5	(0)	0·10
Lee 120 min.	36·0 ± 0·3	33·8 ± 0·7	−2·2	1	4/5	(80)	—
Saline 30 min.	36·5 ± 0·4	34·9 ± 0·7	−1·6	0	4/5	(80)	—
Saline 60 min.	36·7 ± 0·2	32·4 ± 0·6	−4·3	3	5/5	(100)	—
Saline 120 min.	37·0 ± 0·3	34·7 ± 0·9	−2·3	1	4/5	(80)	—

* Deaths considered as — 5·0 °C temperature drop.

** Determined from Chi-square values using Yates correction.

exist, however, between preshock temperature and susceptibility to anaphylaxis. It was noted, furthermore, that the usual clinical indications of anaphylaxis in mice (e.g. lethargy and paralysis) were absent except in those animals showing a depression of body temperature following injection of the shocking material.

A definitive explanation of the modifying effect of these drugs cannot now be offered for lack of direct proof. It is possible that their action may be concerned with their capacity to inhibit $C^1$1, which is activated by antigen-antibody complexes[2]. It is tempting to speculate that the shocked animals may have been refractory to further attempts to produce an anaphylactic response within 24 h in part because of depletion of C^1 1.

Acknowledgment. The technical assistance of Mrs. Dan Myers is gratefully acknowledged.

W. M. Meyers[6] and K. L. Burdon

Department of Microbiology, Baylor University College of Medicine, Houston (Texas), August 18, 1959.

Résumé

L'injection intrapéritonéale chez la souris de cystéine, tyrosine-éthylester ou lysine-éthyl-ester, 1 h avant la production du choc anaphylactique diminue d'une façon significative la sensibilité des animaux à la réaction. Il est possible que cela soit dû à une modification de l'effet du premier composé actif du complément.

[6] Summer Fellow. Allergy Foundation of America, 1957–1958.

Intracellular Peptides of Escherichia coli

One of the ways which could contribute to the elucidation of the mechanism of protein synthesis consists in finding intermediate products of peptide nature. Although free peptides have been detected in various organisms, their relationship to protein synthesis has not been demonstrated conclusively[1–5]. Gram-negative bacteria contain much less intracellular free ninhydrin-positive substances than Gram-positive ones, and glutathione is the only substance of peptidic character which has definitely been detected so far[6]. Further, non-identified ninhydrin-positive substances were described from *E. coli* cells[6] and from the nutrient medium remaining after the growth of these bacteria[7]. Recently, two pure dipeptides were found and identified which accumulate in *E. coli* cells particularly during growth in the presence of chloramphenicol[8].

In further work, other ninhydrin-positive substances from *E. coli B* extract prepared in cold with 5% trichloracetic acid were investigated. This extract of bacteria from 20 l of synthetic medium was treated with ethyl ether to remove trichloroacetic acid, freeze-dried and oxidized with performic acid[9], so that glutathione and possibly other cysteine-containing peptides were present in a single oxidized form. After de-salting on Dowex-50 in the hydrogen form, amino acids and peptides were eluted with ammonia, volume reduced by evaporation and chromatography carried out in a mixture of butanol-acetic acid. In this way free amino acids were separated from other ninhydrin-positive substances which remained at the start of the chromatogram.

After elution, substances from the chromatogram start were separated by high-voltage paper electrophoresis in a pyridine-acetate buffer at pH 5·6 (2·5 h, 29 V/cm)[10].

[1] T. Winnick, R. E. Winnick, R. Acher, and C. Fromageot, Biochim. biophys. Acta *18*, 488 (1955).

[2] L. K. Ramachandran and T. Winnick, Biochim. biophys. Acta *23*, 533 (1957).

[3] H. Borris and G. Schneider, Naturwissenschaften *42*, 103 (1955).

[4] F. Turba and H. Esser, Biochem. Z. *327*, 93 (1956).

[5] G. E. Connell and R. W. Watson, Biochim. biophys. Acta *24*, 226 (1957).

[6] R. B. Roberts, P. H. Abelson, D. C. Cowie, E. T. Bolton, and R. J. Britten, *Studies of Biosynthesis in Escherichia coli* (Carnegie Institution of Washington, Washington D. C. 1955).

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[8] F. Šorm and J. Černá, Coll. Czechoslov. chem. Comm., in press.

[9] B. Keil, Coll. Czechoslov. chem. Comm. *19*, 1006 (1954).

[10] O. Mikeš, Coll. Czechoslov. chem. Comm. *22*, 831 (1957).

This separation produced three weakly basic, one neutral and five acid ninhydrin-positive components. Chromatography in the butanol-acetic acid-pyridine mixture made it possible to resolve the individual components, yielding 30 different ninhydrin-positive substances. These substances proved to be homogeneous on further chromatography in a different solvent system. Hydrolysis in 5 *N* HCl for 18 h at 105°C and chromatography in the butanol-acetic acid mixture provided evidence of the peptide character of these substances.

The isolated peptides contain 3–8 different amino acids, the following occurring most frequently: glutamic acid, cysteine, glycine (i. e. glutathione components), lysine, alanine, and aspartic acid. In isolated cases also arginine, valine, leucine, and threonine were identified. Only with 10 peptides was a sufficient amount obtained to determine the N-terminal amino acids by the dinitrophenyl method[11]. In 5 cases, glutamic acid was found to be the N-terminal amino acid; in other cases aspartic acid, lysine, serine, and alanine (Table).

Table
Intracellular Peptides of *E. coli*

glu. (cys, gly, lys)
glu. (ala, cys, gly, lys)
asp. (cys, gly, lys)
lys. (ala, arg, asp, cys, gly, glu, ser)
asp. (arg, gly, glu, γ-NH_2but, lys, val)
ser. (asp, gly, lys)
ala. (asp, lys)
glu. (ala, asp, cys, gly, lys, leu, val)
glu. (ala, asp, lys, cys, gly)
glu. (cys, gly)

During *E. coli* growth in the presence of chloramphenicol, these peptides can be found intracellularly in larger amounts. The chloramphenicol-resistant strain also contains these ninhydrin-positive substances in a greater degree than the sensitive one.

Metabolic activity and possible participation of these peptides in protein synthesis is being further investigated with the aid of C^{14}-labelled amino acids.

A complete presentation of the work will be published in the Collection of Czechoslovak Chemical Communications.

D. Grünberger, Jiřina Černá and F. Šorm

Cechoslovac Academy of Sciences, Institute of Chemistry, Prague, September 3, 1959.

Zusammenfassung

Mittels Papierchromatographie und Hochspannungselektrophorese konnten in Trichloressigsäureextrakten von *E. coli* 3–8 Aminosäuren enthaltende Peptide isoliert werden, in denen am häufigsten Glutaminsäure, Glycin, Cystein, Lysin, Asparagin und Alanin vorkommt.

[11] F. Sanger and E. O. P. Thompson, Biochem. J. *53*, 353 (1953).

The Rate of Cleavage of β-Mercaptopyruvate by Rapidly Dividing Cells[1]

The enzyme which cleaves β-mercaptopyruvate to pyruvate and atomic sulfur has recently been purified in this laboratory[2]. The same enzyme catalyzes not only the cleavage of the C-S bond but also the transfer of S to an acceptor molecule[3]. The 'physiological' S acceptor as well as the biological importance of this enzyme is unknown. It appears, however, certain that the transsulfurase is one of the key enzymes involved in the anaerobic metabolism of cysteine. The role of thiol compounds in cell division has been often considered although an actual biochemical reaction where SH compounds play a specific role remains to be discovered. A probable exception is glutathione or some acid soluble SH group containing substance which shows quantitative correlation with the rate of mitosis as described by Rapkine[4] and Mazia[5].

We attempted to perform experiments in order to ascertain whether or not the metabolism of β-mercaptopyruvate is correlated with rapid cell division. Quantitative enzyme analyses were made on certain tissues of normal and cancer bearing mice as well as on cancer cells. The choice of the type of cancer cells in such studies is of considerable importance as pointed out by Furth[6] and Klein[7] who suggest that it is preferable to use newly induced tumors. Recently a rapidly growing tumor was induced in pregnant mice by the injection of a suspension of human lung cancer tissue[8,9]. The enzyme content of this tumor and its effect on the host were determined. As shown in the Table, the enzyme content of liver and kidney of normal and tumor bearing animals does not differ significantly. However, the activity of the tumor cells is in every case markedly lower than that of 'normal' tissues. Since the tumors analyzed did not contain necrotic

Table
Rate of pyruvate formation from β-mercaptopyruvate by normal and tumor tissues of mice

No. of Exp.	Liver		Kidney		Tumor
	Normal	Tumor bearing	Normal	Tumor bearing	(Ref. [8,9])
1	6·4	6·0	8·7	15·8	1·3
2	14·5	7·6	17·6	9·0	1·0
3	10·2	8·8	10·7	18·0	1·4
4	7·1	7·4	7·0	8·4	4·2
5	10·1	5·4	11·1	14·6	3·6
6	7·7	7·1	6·7	9·4	2·8
7	12·6	8·9	14·1	13·5	3·2
8	8·7	8·5	—	12·8	1·0
9	6·2	9·4	—	12·6	2·9
10	9·3	11·1	—	17·0	—
11	8·3	7·0	—	8·8	—
	9·2 (± 2·5)	7·9 (± 1·5)	10·8 (± 2·5)	12·7 (± 3·2)	

The results are expressed as 'specific activity' (S.A.) i.e. μmoles of pyruvate formed per 1mg protein per 10 min at 30°C in the presence of 2-mercapto-ethanol. The assay method has been described earlier[2]

[1] Supported by U.S. Public Health Grants H-2897, C-3211 and by a grant of the American Cancer Society (70-4612-24).

[2] E. Kun and D. W. Fanshier, Biochem. biophys. Acta *32*, 338 (1959).

[3] E. Kun and D. W. Fanshier, Biochem. biophys. Acta *33*, 26 (1959).

[4] L. Rapkine, Ann. physiol. physiochim. Biol. *9*, 383 (1931).

[5] D. Mazia, *SH and Growth* in *Glutathione* (A Symposium) (Acad. Press, N. Y.), 1954, p. 209; *The Role of Thiol Groups in the Structure and Functions of the Mitotic Apparatus* in *Sulfur in Proteins* (Acad. Press, N. Y. 1959).

[6] J. Furth, Cancer Res. *19*, 241 (1959).

[7] G. Klein, Cancer Res. *19*, 343 (1959).

[8] C. Klausner and V. Richards, Extr. Bull. internat. Chirurgie *17*, 174 (1958).

[9] V. Richards and C. Klausner, Surgery *44*, 181 (1958).

areas the possibility of an artifact can be excluded. In addition, certain, probably malignant cells grown in tissue cultures also show very low enzymic activity:

(a) unclassified lymph node cells [10] S. A. = 0·33,
(b) cell culture derived from adenocarcinoma of human lung [11] S. A. = 0·53,
(c) Hela cells S. A. = 0·83,
(d) Ehrlich ascites cells S. A. = 0·59,
(e) Rat embryo fibroblasts S. A. = 4·9.

These preliminary observations suggest that the rate of anaerobic metabolism of cysteine is different in normal and in neoplastic cells. The possible biochemical and physiological significance of these observations is being studied.

We acknowledge with thanks the gift of certain tissue cultures received from Miss Virginia Jentoft from the Department of Pathology.

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Department of Pharmacology and Experimental Therapeutics, The University of California Medical Center and Department of Surgery, Stanford University, School of Medicine, San Francisco (Calif.), August 24, 1959.

Zusammenfassung

Quantitative Analysen des β-mercaptobrenztraubensäure-spaltenden Enzyms zeigen, dass Zellen, die sich in aktivem mitotischem Prozess befinden (bzw. Krebszellen und Gewebekulturen), dieses Enzym in viel geringerem Masse enthalten als «normale» Zellen. Da dieses Enzym in dem anaeroben Stoffwechsel von Zystein eine wichtige Rolle spielt, lässt sich vermuten, dass sich der Zysteinstoffwechsel der Tumorzelle quantitativ von dem der Normalzelle unterscheidet.

[10] S. D. Bell, Jr. and R. E. Johnson, Proc. Soc. exp. Biol. Med. *92*, 46 (1956).
[11] A. W. Frisch, V. Jentoft, R. Berger, and E. J. Loosli, Amer. J. clin. Pathol. *25*, 1107 (1955).
* Established Investigator of the American Heart Association. Inc., New York.

On a Glycoprotein of the Sea Urchin Eggs and its Changes Following Fertilization

Shaking with ether the trichloroacetic acid (TCA) soluble fraction of the unfertilized egg of *Paracentrotus lividus* results in the formation of a fluffy precipitate at the interface between the water and the ether phases. When the same procedure is applied to the TCA-soluble fraction of newly (5 min) fertilized eggs or developmental stages, no such precipitate appears. The analysis of the fluffy precipitate has given the following results (as % of dry weight):

Nitrogen:	5·6–7·4
Carbohydrates:	43·0–52·7
Glucosamine:	1·6

No phosphorus has been found and 2 h extraction with boiling alcohol-ether (3:1) resulted in no change in dry weight.

Only one carbohydrate, glucose, was identified by paper chromatography and this is considered as a good evidence that the fluff is not a contamination from material of the jelly-coat incompletely removed. In fact, the main carbohydrate component of the jelly-coat of *Paracentrotus* is fucose (Minganti and Vasseur[1]). The following amino acids have also been identified by two dimensional paper chromatography of the acid hydrolysate of the fluff: cyst-(e)ine, arginine, lysine, histidine, aspartic acid, glutamic acid, glycine, serine, alanine, proline, methionine, valine, threonine, phenylalanine, leucine, iso-leucine.

In a second series of experiments the total polysaccharides present in the TCA soluble fraction of unfertilized and newly fertilized eggs were precipitated with alcohol. Equal amounts of eggs were used in each one of three parallel sets of experiments. The results given in the Table indicate that the amount of alcohol precipitable material has significantly decreased after fertilization and that an even greater decrease in the *N* content of the precipitate takes place.

Here again the only carbohydrate present was glucose and it made up almost the total dry weight of the precipitate.

On the other hand determinations of the total (free + bound) carbohydrates present in the TCA soluble fraction failed to show any significant change as a result of fertilization. The average of six determinations was (as % of the total carbohydrates of the eggs), 30 ± 1·47 in the unfertilized eggs and 27·2 ± 1·29 in those newly fertilized, $t = 0{\cdot}89$, $P = 0{\cdot}4$. It must be added that in four different determinations we have also been unable to detect any change in the total carbohydrate content of the eggs following fertilization.

Table

Amount of alcohol-precipitable material from the TCA-soluble fraction from equal amounts of unfertilized and newly fertilized eggs of *Paracentrotus lividus*

Exp. No.	mg (dry weight) of precipitate		% total N	
	Unfertilized	Fertilized	Unfertilized	Fertilized
1	12·0	8·8	1·35	0·62
2	10·4	8·5	2·20	1·35
3	15·9	12·5	1·93	1·21

The most likely interpretation of these results seems to be that upon fertilization some change takes place in a glycoprotein fraction present in the unfertilized egg as a result of which it becomes no longer precipitable either by the treatment with ether or alcohol.

By applying the Hotchkis method, Runnström and Immers[2] have described a staining of the outer rim of the cortex of the unfertilized egg. 5 min after fertilization this continuous layer is broken up into a great number of small granules which a little later become incorporated into the hyaline layer. This observation agrees well with our own results as it gives histological evidence of a change of some kind in a cortical glycoprotein of the egg upon fertilization.

[1] A. Minganti and E. Vasseur, Acta Embryol. Morphol. exper. *2*, 195 (1959).
[2] J. Runnström and J. Immers, Exper. Cell Res. *10*, 354 (1956).

Experimental. Eggs of *Paracentrotus lividus* were washed several times with sea water, made up to an exact volume and then divided into two equal portions, one of which was fertilized and collected 5 min after the beginning of the elevation of the fertilization membrane while the other one was processed unfertilized. The jelly coat was removed by the usual treatment with acidified (pH 5·0) sea water, the eggs packed by a quick centrifugation and then suspended and homogenized in cold 10% TCA. Samples were withdrawn for the determination of total nitrogen and carbohydrates and then the homogenate was centrifuged at 12000 *g* in the cold for 10 min. The supernatant was collected and samples taken for the determination of total carbohydrates. The TCA-soluble fraction was then shaken 3 to 5 times with an equal volume of ether in a glass-stoppered cylinder. After the last shaking, the ether was withdrawn and the water phase with the fluffy layer at its surface was decanted to a centrifuge tube and left overnight in the refrigerator. Upon centrifugation, the fluffy layer collected at the surface: using a thin stemmed pipette which reached the bottom of the tube and by carefully regulating the suction of a water pump connected to it, the water phase could be withdrawn almost entirely. The fluff was then suspended in 50% alcohol and sedimented by centrifugation; this washing was repeated five times. The precipitate was then washed three more times with 95% alcohol, twice with 3:1 alcohol-ether, ether-alcohol (3:1), ether and dried.

For the preparation of the alcohol-precipitable components, two volumes of cold 95% ethanol were slowly added, with continual stirring, to one volume of the TCA soluble fraction in a centrifuge tube. After standing overnight in the refrigerator, the precipitate was centrifuged, the supernatant pipetted off, the precipitate resuspended in a small amount of 50% alcohol and transferred quantitatively to a smaller tube in which it was washed and dried as described above.

For the chromatographic analysis of carbohydrates, the material was hydrolyzed in 0·5 *N* H_2SO_4 at 110°C for about 12 h and the hydrolysate neutralized, desalted (by treatment with IR-B 4 and IR-120 Amberlites) dried, redissolved in a very small amount of water and chromatographed on Whatman No. 1 paper. The chromatogram was irrigated for 24 h with butanol-acetic acid-water (3:1:1) and developed with anilin-phtalate (Partridge[3]). For the quantitative determination of carbohydrates, a known amount of material was hydrolyzed as indicated above and the hydrolysate was neutralized, brought to volume and aliquotes were used for the analysis according to Park and Johnson[4] using glucose as a reference. The anthrone method (Hewitt[5]) has been used in some cases. The chromatographic analysis of the amino acids was done on material hydrolysed with 6 *N* HCl and desalted by passage through a Dowex-50 column in the acid cycle. The two dimensional paper chromatography was run according to Redfield[6]. Glucosamine was determined according to Gardell[7]. Nitrogen was determined by Nesslerization after combustion and phosphorus according to Allen[8].

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A. Monroy and M. L. Vittorelli

Istituto di Anatomia comparata, Università di Palermo, August 3, 1959.

Riassunto

Sbattendo con etere la frazione solubile in acido tricloroacetico di uova vergini di *Paracentrotus lividus* si forma un precipitato all'interfacie tra etere e fase acquosa. L'analisi di questo precipitato ha dimostrato che si tratta di una glicoproteina. Il precipitato non si forma da estratti di uova fecondate.

Étude immunologique des Haptoglobines humaines individuelles

Les Haptoglobines individuelles[1] se manifestent dans le sérum humain sous forme de trois phenotypes principaux (Hp 1–1, Hp 2–2 et Hp 2–1) correspondant à deux types I et II d'haptoglobine.

Ceux-ci ont pu être identifiés à une forme monomère ou dimère de l'haptoglobine[2] et présentent à l'ultracentrifugation des constantes de sédimentation différentes[3]. L'étude immunologique de ces constituants (Bearn et Franklin[3]) par les courbes de précipitation spécifique entre antisérums anti-haptoglobine de chaque type et ces haptoglobines a conclu à une identé antigénique de ces constituants.

Cette note a pour objet de rapporter les résultats de nos propres expériences concernant l'étude antigénique des types d'haptoglobine.

Protocole expérimental

L'étude du comportement immunoélectrophorétique de chaque type de sérum a été réalisée suivant les modalités décrites par Grabar et Williams[4], soit avec un sérum de cheval anti-sérum humain total, soit avec des sérums de lapins préalablement immunisés par des sérums humains de différents types (anti-Hp 1–1, anti-Hp 2–1, anti-Hp 2–2).

La coloration spécifique du complexe haptoglobine-hémoglobine dans les lignes de précipitation était réalisée par la technique que nous avons décrite avec Uriel[5].

Enfin, les expériences de double diffusion en gélose ont été réalisées, soit par la méthode d'Ouchterlony[6] soit par la variante de Kaminski[7].

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[5] J. M. Fine, J. Uriel et J. Faure, Bull. Soc. Chim. biol., Paris *38*, 649 (1956).
[6] O. Ouchterlony, Acta path. microbiol. scand. *26*, 507 (1949).
[7] M. Kaminski, Biochim. biophys. Acta *13*, 216 (1954).

[3] S. M. Partridge, Nature *164*, 443 (1949).
[4] J. T. Park and M. J. Johnson, J. biol. Chem. *181*, 149 (1949).
[5] B. R. Hewitt, Nature *182*, 246 (1958).
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[7] S. Gardell, Acta chem. scand. *7*, 201 (1953).
[8] R. J. L. Allen, Biochem. J. *34*, 858 (1940).

Résultats

A. – *Electrophorèse en gélose et Immunoélectrophorèse.* Après électrophorèse en gélose, les complexes de type Hp 1–1 ont une mobilité plus élevée que ceux de type 2–2, la position des complexes de type Hp 2–1 étant intermédiaire entre les deux précédents. Les lignes de précipitation spécifiques entre les anticorps anti-haptoglobine et ces complexes traduisent encore plus nettement ces différences de mobilité électrophorétique par l'emplacement de centre de l'arc de courbure de la ligne de précipitation colorée à la benzidine (Fig. 1). Deux lignes donnent la réaction à la benzidine dans le sérum humain quelque

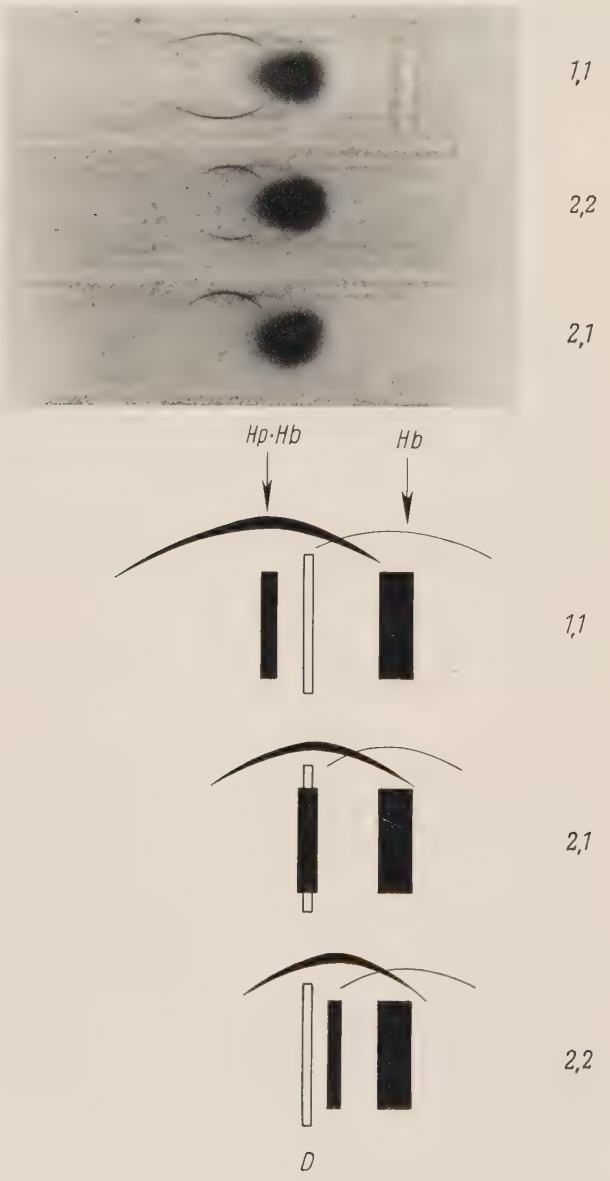

Fig. 1. – Immunoélectrophorèse de sérums humains de type Hp 1–1, Hp 2–1 et Hp 2–2 additionnés d'hémoglobine. Les lignes de précipitation des complexes Hb/Hp sont révélés par la réaction à la benzidine

soit le groupe d'haptoglobine. Une ligne, de mobilité α_2, correspond au complexe Hp/Hb, la deuxième de mobilité β_1 correspond à l'hémoglobine résiduelle entrainée dans la précipitation d'une β_1 globuline.

Des expériences réalisées sur 10 échantillons de sang de cordon montrent, non pas une absence totale d'haptoglobine, mais une ligne α_2 donnant une réaction faible à la benzidine, et ayant une position analogue aux haptoglobines adultes de type Hp 1–1.

Des expériences analogues ont été réalisées, non plus en tampon véronal de pH 8,2 mais en tampon phosphate de pH 7. Les sérums de type Hp 2–1 et Hp 2–2 montrent une ligne de précipitation à double courbure deux fois plus étendue que la ligne correspondant aux sérums de type Hp 1–1.

B. – *Double diffusion en gélose.* Comme on peut le voir sur la Figure 2a il est possible de dénombrer, en plus de la ligne correspondant à l'hémoglobine résiduelle, deux lignes de précipitation colorables par le réactif à la benzidine dans les sérums de types Hp 2–2 et Hp 2–1 et une seule ligne de précipitation dans les sérums de type Hp 1–1. La ligne unique de précipitation observée chez les sujets du type Hp 1–1 donne une réaction d'identité complète avec une des lignes des sujets de types Hp 2–1 et Hp 2–2 (Fig. 2b).

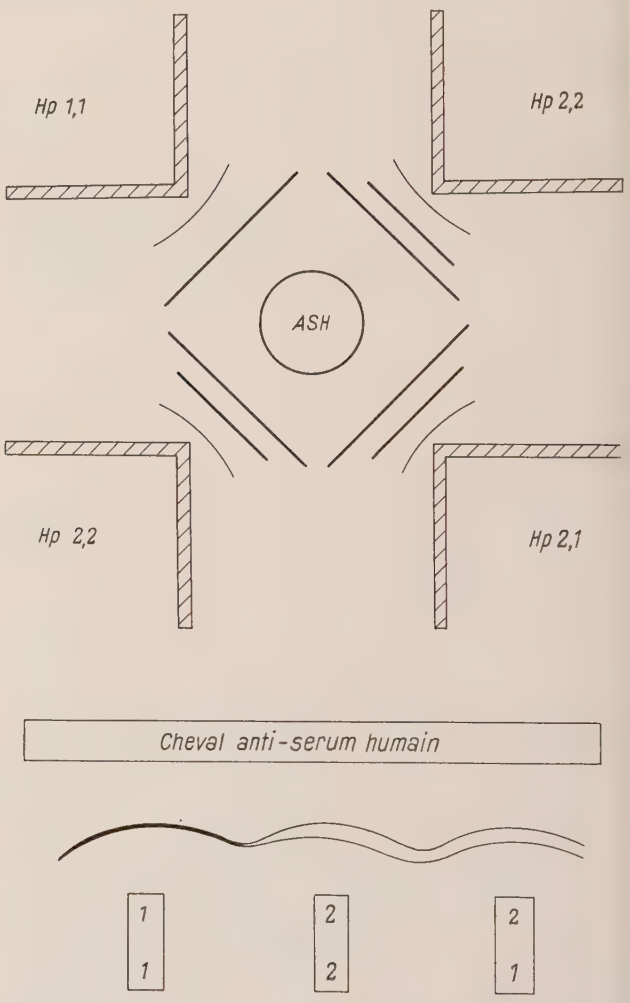

Fig. 2. – Double diffusion en gélose de sérums de différents types d'haptoglobine et d'un sérum de cheval anti-sérum humain (Institut Pasteur): *a*) Méthode d'Ouchterlony; *b*) méthode de Kaminsky montrant l'identité antigénique des trois types d'haptoglobine

Les sangs de cordon ne montrent qu'une seule ligne de précipitation colorable à la benzidine. Cette ligne montre une identité antigénique complète dans les 10 échantillons de sangs de cordon examinés. Par rapport aux lignes de

précipitation données par les groupes de type 1–1, 2–1 et 2–2, cette ligne se situe entre la ligne commune entre les Hp 1 et les Hp 2, et la ligne supplémentaire observée chez les sujets de type 2–1 et 2–2.

C. – *Expériences d'épuisements d'anti-sérums de lapins anti-sérums de différents type d'haptoglobine.* Des sérums de lapins anti-sérum humain de type Hp 1–1 épuisés par du sérum humain de type Hp 2–2 ne donnent plus de réaction de précipitation avec aucun sérum des trois types. Inversement, des sérums de lapins anti-sérum humain de type Hp 2–2 épuisés par du sérum humain de type Hp 1–1 ne réagissent plus en double diffusion contre les sérums des trois types.

Conclusion

Comme Bearn et Franklin, nous pensons à la suite de ces expériences que les trois types d'haptoglobine ont une communauté antigénique indiscutable comme en font foi la ligne de précipitation commune aux trois types de sérum et les expériences d'épuisement des anti-sérums.

On peut cependant soulever l'hypothèse devant l'existence d'une deuxième ligne de précipitation dans les sérums de type 2–1 ou 2–2, soit d'une différence antigénique partielle entre les complexes de l'haptoglobine dimère et ceux de l'haptoglobine monomère, soit de l'extériorisation de phénomène de diffusion distincts entre deux parties du même complexe (dépolymérisation partielle de l'haptoglobine dimère).

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Centre National de Transfusion Sanguine, Paris et Clinique Pédiatrique de Parme (Italie), le 20 juillet 1959.

Summary

Immunological studies of the haptoglobin-hemoglobin complexes of different serum groups show different mobility by immuno-electrophoretic technique, and the antigenic relationship of these haptoglobins.

Zur Wirkungsweise einiger Katalase-Inhibitoren

Eine grosse Anzahl von Verbindungen der verschiedenartigsten Struktur hemmen die Katalase. Drei anorganische Partikel zeichnen sich durch besonders hohe Wirksamkeit aus: CN^-, N_3^- und SH^- [1]. Von diesen Verbindungen ist zugleich bekannt, dass sie im Tierversuch gegen harte Strahlung einen gewissen Schutz auszuüben vermögen[2]. Aus verschiedenen Arbeiten[3] geht hervor, dass im Mechanismus der chemischen Strahlenschutzwirkung die Katalase eine bedeutende Rolle spielt. Es war daher von Interesse, zu untersuchen, auf welchem Wege die Katalasehemmung durch CN^-, N_3^- und SH^- zustande kommt.

[1] E. Boyland und E. Gallico, Brit. J. Cancer *6*, 160 (1952).

[2] Z. M. Bacq und H. Hervé, Brit. J. Radiol. *24*, 617 (1951). – N. W. Lutschnik und J. A. Timofejewa-Ressowskaja, Ber. Akad. Wiss. UdSSR *116*, 407 (1957). – G. Guidotti und F. Passalacqua, Farmaca, Ediz. sci. *10*, 214 (1955). – H. P. Riley, Genetics *42*, 593 (1957). – Z. M. Bacq, H. Mugard und A. Hervé, Acta radiol. *38*, 489 (1952).

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Die drei erwähnten Partikel können sich als starke einzähnige Komplexliganden betätigen. Von Komplexen des Ferrihämoglobins, das der Katalase strukturell nahesteht, mit diesen Liganden L sind Stabilitätskonstanten bekannt[4] (Tab.).

Tabelle

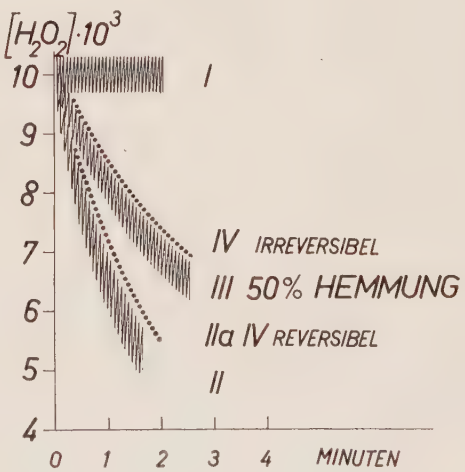
L	log K	5%ige Katalasehemmung[1]
CN^-	7,2	$6 \cdot 10^{-6}$ M
N_3^-	5,1	$4 \cdot 10^{-7}$ M
SH^-	5,3	$8 \cdot 10^{-6}$ M
CNS^-	2,5	$> 10^{-1}$ M
F^-	2,2	$> 10^{-1}$ M
Imidazol	2,6	$> 10^{-1}$ M

Ein Vergleich der Stabilitätskonstanten mit der Ligandenkonzentration, die eine 50%ige Hemmung hervorzurufen vermag, legt den Gedanken nahe, dass die Hemmreaktion primär durch Besetzung einer Koordinationsstelle am zentralen Fe der Katalase zustande kommt. Es wird dadurch die Ausbildung des aktiven «compound I»[5] verhindert. Wenn dies der Fall ist, dann muss – mindestens für kurze Zeit – ein reversibles Komplexbildungsgleichgewicht (1) existieren, und durch Zugabe geeigneter Partikel, zum Beispiel Ag^+, müsste sich dieses Gleichgewicht wieder nach links verschieben lassen (2), was notwendigerweise zu einer Enthemmung der Katalase führt.

$$\text{Katalase} + L \rightleftharpoons [\text{Katalase} \cdot L]\ \text{gehemmt} \quad (1)$$

$$[\text{Katalase} \cdot L] + Ag^+ \rightleftharpoons \text{Katalase}\ \text{enthemmt} + AgL^+ \quad (2)$$

Um diesen Sachverhalt für die Liganden CN^-, N_3^- und SH^- zu überprüfen, bedienten wir uns für die Aktivitätsbestimmungen der polarographischen Methode, die zur Messung der anfänglichen hohen «α-Aktivität» der Katalase[6] besonders geeignet ist. H_2O_2 zeigt bei $-1{,}1$ Volt eine Reduktionsstufe, die quantitativ gut auswertbar ist. Wir bestimmten die Stufenhöhe bei $-2{,}0$ Volt. Bei konstant gehaltener Spannung liefert das Polarogramm direkt eine kontinuierliche H_2O_2-Konzentrations-Zeitkurve (Abb.).



Polarographische Verfolgung der $[H_2O_2]$ (Hg-Tropfelektrode-Ag/AgCl), $E = -2{,}0$ V konstant

[4] C. D. Coryell, *Chemical Specificity in Biological Interactions* (New York 1954), p. 108.

[5] D. Keilin und D. Nichols, Biochim. biophys. Acta *29*, 302 (1958).

[6] P. George, Biochem. J. *44*, 197 (1949).

Alle Versuche führten wir in 0,05 M Phosphatpuffer vom pH 7 durch. Die Temperatur betrug 20± 2°C. Die anfängliche H_2O_2-Konzentration betrug immer 10^{-2}. Zur Versuchszeit 0 wird auf ein Endvolumen von 20 ml 1 ml einer 4×10^{-4}%igen frisch bereiteten Lösung von kristallisierter Katalase Boehringer zugegeben. Die anfängliche Steilheit der $[H_2O_2]$-Zeitkurve nahmen wir als Mass für die α-Aktivität der Katalase[6].

Mit dieser Messanordnung führten wir nun mit den drei Hemmstoffen CN^-, N_3^- und SH^- eine analoge Versuchsreihe durch. In Versuch I wird zunächst die Reinheit der H_2O_2-Lösung sowie die polarographische Apparatur und in Versuch II die α-Aktivität der Katalase kontrolliert. In Versuch III wird unmittelbar nach der Katalase soviel Inhibitor zugesetzt, dass eine 50%ige Hemmung eintritt. In Versuch IIa wird vor dem Hemmstoff ausserdem noch Ag^+ zugesetzt und kontrolliert, ob die $[Ag^+]$ ausreicht, um den Hemmstoff zu binden. In Versuch IV, der entscheidet, ob eine Enthemmung im Sinne von (2) möglich ist, wählten wir die umgekehrte Reihenfolge der Zugabe: Katalase, Hemmstoff, Ag^+.

Es zeigte sich, dass sich bei Versuchen vom Typus IV die drei Hemmstoffe grundsätzlich verschieden verhalten. Die einmal durch CN^- gehemmte Katalase kann wieder reaktiviert werden durch Zugabe von Ag^+. Die CN^--Hemmung ist somit, im Einklang mit den Versuchen von Chance[7], primär auf eine reversible Komplexbildungsreaktion zurückzuführen. Hingegen kann die durch SH^- und N_3^- gehemmte Katalase nicht wieder enthemmt werden, was dafür spricht, dass hier ein ganz anderer Mechanismus wirksam ist oder dass nach der Komplexbildung sofort eine Folgereaktion eintritt[8], die den Vorgang irreversibel macht.

Fräulein Lucia Graf danken wir für die Durchführung der Katalaseversuche. Der Stiftung zur Förderung der Arbeiten auf dem Gebiete der Atomwissenschaften danken wir bestens für die gewährte Unterstützung.

S. Fallab und H. Erlenmeyer

Anstalt für anorganische Chemie, Basel, 19. November 1959.

Summary

The mechanism of catalase inhibition by CN^-, N_3^-, and SH^- has been investigated by a polarographic method. CN^- has been found to inhibit reversibly by a coordinating mechanism, whereas N_3^- and SH^- inhibits catalase irreversibly.

[7] B. Chance, J. biol. Chem. *179*, 1299 (1949).

[8] In Übereinstimmung mit den Versuchen von D. Keilin und D. Nichols, wonach N_3^- peroxydatisch oxydiert wird[5].

On the Protease and Amylase Production in the Midgut Gland of Young and Mature African Snails

The African Giant Snail, *Achatina fulica* Bowdich, changes its diet and feeding habits markedly as it grows older: whereas young snails feed mainly on fresh leaves and other green parts of plants, the older, mature snails are noted for their omnivorous feeding habits. They usually feed on decaying matter, even on small dead animals[1,2]. Such an obvious change in feeding habits suggests the possibility of a change in the enzyme pattern of the large midgut gland, in that young snails may produce more carbohydrates and probably less proteases than older ones.

To check this hypothesis 'enzyme powders' were made of glands from young (shell-length less than 4 cm) and older snails (shell-length 7 cm and over) which had previously beens starved for 1–2 weeks, during which time feces was removed regularly. Water was available to them all the time to prevent estivation. The enzyme activity of the powders was determined (see for the technique used of making enzyme powders, the enzyme extracts and the determination of the amylase and protease activity 7 and 8). The results of these experiments were (Table):

Table

(a) *Amylase* (amount of reducing sugars present, expressed in mg glucose)

Small snails	Large snails
70·4	70·4
73·4	70·4
73·1	69·2
73·2	70·4
	70·4

(b) *Protease* (Sørensen's formaldehyde titration of 2 ml of enzyme extract-substrate mixture)

0·02	0·22
0·15	0·21
0·15	0·17
0·09	0·21
0·10	0·18
	0·26
	0·18

The significance of the differences in the population of data for amylase and protease activity between the young and the old snails was statistically tested by applying a *t*-test for small samples of unequal size[3]. The *p*-values for amylase- and protease activity in both groups are 0·02 and approximately 0·003 respectively. Hence, there is a significant difference in the activities of these two enzymes between the groups: *in the herbivorous snails slightly more amylase is produced than in the old omnivorous ones. The latter produce definitely more proteases.*

The changes of the enzyme activity in *Achatina* as it grows older agree very well with the changes observed in its feeding habits: the enzyme production 'fits' in the changed (normal) diet. The question now arises: is this change in enzyme production caused by that in feeding habits, or is it an integrative part of this latter change? Individual, diet depending changes in the digestive enzyme production have been reported to exist in a number of cases[4–6], but such clear-cut correlative changes were not demonstrated to exist in the older *Achatina*[7–9]. It seems

[1] P. B. van Weel, Chron. Nat. *104*, 241 (1948).

[2] H. W. Lange, Pac. Scie. *4*, 323 (1950).

[3] We are indebted to Dr. A. L. Tester, who kindly checked the results.

[4] M. I. Grossman, H. Greengard, and A. C. Ivy, Amer. J. Physiol. *138*, 676 (1942/43).

[5] B. T. Squires, J. Physiol. *119*, 153 (1953).

[6] W. E. Knox, V. H. Auerbach, and E. C. C. Lin, Physiol. Rev. *36*, 164 (1956).

[7] C. L. Prosser and P. B. van Weel, Physiol. Zool. *31*, 171 (1958).

[8] P. B. van Weel, Z. vgl. Physiol., in print (1959).

[9] P. B. van Weel, Exper. *15*, 110 (1959).

therefore logical to assume that in this snail the pattern of enzyme production changes on ageing. Whether this affects the food preference, or whether it is only a part of the entire physiological process, leading to a change in feeding habita, is still an unsolved problem.

A. C. Smith and P. B. van Weel

Department of Zoology and Entomology, University of Hawaii (U.S.A.), September 16, 1959.

Zusammenfassung

Junge Individuen von *Achatina fulica* Bowdich fressen mit Vorliebe Pflanzenteile, ältere Individuen sind omnivor. Die Enzymaktivitäten der grossen Mitteldarmdrüse wurden untersucht: bei jungen Tieren wird etwas mehr Amylase als bei älteren Individuen, bei älteren Tieren deutlich mehr Protease als bei jüngeren Individuen produziert. Zwischen Enzymproduktion und Futterwahl besteht also eine Korrelation, doch ist ihre kausale Beziehung noch nicht geklärt.

Alkaloid Studies XXIII[1]
Isolation of Four New Aspidosperma Alkaloids: Cylindrocarpine, Refractine, Pyrifoline, and Pyrifolidine

The recent interest[2] in alkaloids of the genus *Aspidosperma* prompts us to record in preliminary form some of the results of a systematic survey of Brazilian *Aspidosperma* species which is now in progress in our laboratories.

Our results with *A. olivaceum* Muell.-Arg. do not differ from those already published recently[3], while the only

[1] Paper XXII, J. S. E. Holker, M. Cais, F. A. Hochstein and C. Djerassi, J. org. Chem. *24*, 314 (1959).

[2] For leading references see: J. Schmutz and H. Lehner, Helv. chim. Acta *42*, 874 (1959). – P. C. Ferreira, G. B. Marini-Bettolo, and J. Schmutz, Exper. *15*, 179 (1959). – M. A. Ondetti and V. Deulofeu, Tetrahedron Letters No. 7, 1 (1959).

[3] J. Schmutz and F. Hunziker, Pharm. Acta Helv. *33*, 341 (1958).

Table

	Cylindrocarpine		Refractine		Pyrifoline		Pyrifolidine		
Formula	$C_{29}H_{32}N_2O_4$		$C_{22}H_{26}N_2O_4$		$C_{23}H_{30}N_2O_3$		$C_{22}H_{30}N_2O_3$	$C_{23}H_{32}N_2O_3$	
Analysis	Calc.	Found	Calc.	Found	Calc.	Found	Calc.	Calc.	Found
C	73·70	73·72	69·09	69·34	72·22	72·24	71·32	71·84	71·70
H	6·83	6·86	6·85	6·88	7·91	7·91	8·16	8·39	8·27
N	5,93	5·73	7·32	7·33	7·32	7·43	7·56	7·29	7·50
O	13·54	13·77	—	—	12·55	12·64	12·96	12·48	12·79
2 OCH_3	13·13	13·06	16·23	16·16	16·25	16·07	16·76	16·14	16·77
N-acetyl	—	—	11·25	11·65	11·25	11·15	11·62	11·20	11·25
Mol-weight [a]	—	—	382·5	378	382·5	373	370·5	384·5	374
M. p.	168–169° C		157·5–159° C		141·5–143·5° C		147·5–150° C		
$[\alpha]^b$	−181°		−23·5°		+102°		+90°		
pKa[c]	5·9		6·64		6·40		6·85		
Ultraviolett absorption spectrum[d]	λ_{max} mμ	log ε	λ_{max} mμ	log ε	λ_{max} mμ	log ε	λ_{max} mμ		log ε
	216	4·53	216	4·43	217	4·41	223		4·50
	248	4·05	260	4·12	261	4·04	252		3·94
	285	4·31	286–289 inflection	3·44–3·45	286–288 inflection	3·63	286		3·32
	λ_{min}	log ε	λ_{min}	log ε	λ_{min}	log ε	λ_{min}		log ε
	237	4·02	237	3·78	238	3·73	243		3·91
	254	4·04					279		3·31
Infraredabsorption spectrum [e]	λ_{max} μ		λ_{max} μ		λ_{max} μ		λ_{max} μ		
	5·79		5·74		6·10		6·05		
	6·06		6·00		6·23		6·14		
	6·23		6·27		6·29				

[a] Perchloric acid titration; [b] All rotations in chloroform; [c] Potential titration in 66% dimethylformamide; [d] In ethanol; [e] In chloroform

pure alkaloid encountered by us in *A. pyricollum* Muell.-Arg. proved to be ulein[4], as demonstrated by direct comparison with a specimen kindly provided by Dr. J. Schmutz. The following three species yielded several new alkaloids, of which four have been isolated in pure form. The empirical formulas given below are consistent with the analytical data, but they should only be considered as tentative ($\pm CH_2$) until more extensive degradative work has been completed.

Aspidosperma cylindrocarpon Muell.-Arg. furnished *cylindrocarpine* (Table) after purification through the crystalline perchlorate and regeneration of the alkaloid. All oxygen functions are accounted for by a N-cinnamoyl-7-methoxy-dihydroindole grouping and one carbomethoxy function. Their presence was established by acid hydrolysis (yielding cinnamic acid), by hydrogenation to dihydrocylindrocarpine (yielding dihydrocinnamic acid), whose ultraviolet absorption spectrum was practically superimposable upon that of aspidospermine[5], and by alkaline hydrolysis of dihydrocylindrocarpine to the amino acid dihydrocylindrocarpic acid, which could be remethylated with diazomethane to dihydrocylindrocarpine.

Aspidosperma refractum Mart. extract gave after chromatography of the weakly basic fraction *refractine* (Table). The extreme similarity of the ultraviolet absorption spectrum with that of aspidospermine coupled with the N-acetylanalysis indicates the presence[5] of a N-acetyl-7-methoxy-dihydroindole moiety, while the infrared band at 5·74 μ, the methoxyl analysis and the saponification results show that the other two oxygen atoms form part of a carbomethoxy group.

Aspidosperma pyrifolium Mart. furnished the two alkaloids *pyrifoline* and *pyrifolidine* which were separated by fractional crystallization of the perchlorates from the nonphenolic alkaloid fraction. The analytical, spectral and infrared data of pyrifoline indicate the presence of a N-acetyl-7-methoxy-dihydroindole chromophore and of an aliphatically bound methoxyl function.

Work is now under way in our laboratories on the structure elucidation of these alkaloids as well as on the isolation of additional alkaloids from the above-mentioned *Aspidosperma* species.

Acknowledgement: Financial support for these investigations has been provided by grants H-2574 and H-4944 of the National Heart Institute (U. S. P. H. S.), by Eli Lilly & Co. (Indianapolis, Ind.) and by a grant from the Conselho Nacional de Pesquisas, Brazil. We are also indebted to the physical chemistry staff of Eli Lilly & Co. for the potentiometric titrations. The plant collections were principally arranged by Dr. W. B. Mors (Instituto de Quimica Agricola, Rio de Janeiro) in collaboration with Mr. Plinio de Castro Prado, Dr. C. T. Rizzini, Dr. Dórdano de A. Lima and the Comisão do Vale de São Francisco. We are indebted for botanical determinations to Dr. Fr. Markgraf, Zurich Botanical Garden, and Drs. Afrício Duarte and Armando de Matos Filho, Rio de Janeiro Botanical Garden.

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[4] J. Schmutz, F. Hunziker, and R. Hirt, Helv. chim. Acta *40*, 1189 (1957). – G. Büchi and E. W. Warnhoff, J. Am. Chem. Soc. *81*, 4433 (1959).

[5] The presence of a N-acetyl-7-methoxy-dihydroindole chromophore in aspidospermine was demonstrated by J. R. Chalmers, H. T. Openshaw, and G. F. Smith, J. chem. Soc. *1957*, 1115.

[6] Postdoctorate research fellow 1958–1959 (Wayne State University); 1959–1960 (Stanford University).

Zusammenfassung

Die Isolierung von vier neuen *Aspidosperma*-Alkaloiden ergab *Cylindrocarpin* (erstes N-Cinnamoyl-dihydroindol-derivat) sowie *Refractin*, *Pyrifolin* und *Pyrifolidin* (alles N-Acetyl-dihydroindole), die alle vier eine Methoxyl-gruppe im Dihydroindolsystem besitzen.

Triterpene Acids from *Commiphora glandulosa* Schinz[1]

The chemistry of the genus *Commiphora* Jacq. (= *Balsamodendron* Kunth), natural order Burseraceae, has not been extensively studied; earlier references are given by Wehmer[2]. Myrrh, usually obtained from *C. abyssinica* (Berg) Engl., *C. molmol* Engl. and *C. opobalsamum* (L.) Engl., has been found to consist of volatile oils, mostly mono- and sesquiterpenes, gums, and unidentified resins, but the presence of triterpenes has never been suggested[3], and it was therefore of interest to find that the resin of *C. glandulosa* Schinz[4], a tree growing in the arid parts of Southern Africa, is a rich source of triterpene acids, both free and combined as glycosides. From two samples of resin collected for us by Dr. I. B. Pole Evans at different times from the same tree, we found that the free acids were present in roughly the same proportions, but that the glycosides in one case yielded mainly 'aglycone E' and in the other 'aglyconec' (see below for nomenclature).

The resin of *C. glandulosa* is soluble in cold ether to an extent of about 50%. The free acids (about 25% of original resin) were isolated from the ethereal solution through the barium salts, then methylated with diazomethane, and the methyl esters separated on a column of neutral alumina (activity III, according to Brockmann[5]). Extraction of the residual resin with ether or methanol at reflux temperature yielded the crude crystalline glycosides (about 10% of original resin).

Five methyl esters could be separated by this method; these will be designated methyl commate A, B, C, D, E, following the order in which they were eluted, graded solvents from ether-chloroform (1:1) through chloroform to chloroform-methanol (9:1) being used. Esters A, B, and C were purified by recrystallization from methanol, and E was recrystallized from chloroform. Ester D could not be freed from C using methanol as a solvent, but recrystallization from methylene chloride, in which C dissolves readily, gave an apparently homogeneous product.

[1] We are indebted to Dr. H. Hürlimann for supplying the botanical information in this paper.

[2] C. Wehmer, *Die Pflanzenstoffe*, 2. Aufl., II (Jena 1931), p. 647.

[3] Triterpenes are well known from other genera of the order Burseraceae, e.g. *Canarium* (Elemi resin), *Boswellia* etc.

[4] *C. glandulosa* belongs to the group of *C. pyracanthoides* Engl. s. lat., as pointed out by J. P. M. Brenan, Kew Bull. *1953*, 104. This author regards *C. lugardae* N. E. Br., *C. seineri* Engl. and *C. berberidifolia* Engl. as synonyms of *C. glandulosa*. The species seems to be distributed over a wide area of Southern Africa, from Mozambique through the Rhodesias to Bechuanaland, Transvaal, and South-West Africa. – It is noteworthy that we found the resins of *C. roxburghii* (Stocks) Engl. (= *C. mukul*) and of *C. viminea* Burtt-Davy, a species still somewhat related to *C. pyracanthoides* (Brenan), to be completely devoid of triterpene acids both free and combined as glycosides.

[5] H. Brockmann and H. Schodder, Ber. dtsch. chem. Ges. *74*, 73 (1941).

The IR. spectra (Table IV) and the physical properties (Tables I–III) indicated that the compounds were distinct and different. Homogeneity of the esters was checked by chromatoplate chromatography[6] developing with chloroform-ethyl acetate (4:1) and detecting with concentrated sulphuric acid. Single spots were observed in the case of each of the five esters, R_f values being 0·70 for esters A and B, 0·18 for esters C and D, and 0·07 for ester E. In this solvent system betulinic, oleanolic, and ursolic acid methyl esters ran with the solvent front. Methoxyl determination on esters B–E showed that these were monoesters of C_{30} acids. The properties of the esters are given in Table I, together with the approximate percentage in which they occurred in the original acid mixture.

Acetylation of the methyl esters in pyridine gave the methyl ester acetates, the properties of which are listed in Table II.

Reduction with lithium aluminium hydride in ether or tetrahydrofuran (for methyl commate-A and -E) gave the corresponding alcohols, the commols, the properties of which are shown in Table III.

[6] E. Demole, J. Chromatography *1*, 24 (1958). – We are indebted to Mr. E. von Arx for carrying out these determinations.

All methyl commates gave a positive tetranitromethane test in chloroform except methyl commate-E which is very sparingly soluble in this solvent; its diacetate was positive in the test. The optical rotational values of the methyl esters and their acetates, the high melting points of all the compounds (particularly the methyl esters) and the results of the combustion analyses strongly suggest that these compounds must be classed with the pentacyclic triterpenes.

Methyl commate-A is considered to be an artefact arising by overmethylation of another ester. This suggestion is made to account for the following facts. In contrast to esters B–E, which occurred in about the same percentages in all the batches worked up, the content of ester A varied from 0–1·0% of the mixture. Methyl commate-A contains one acetylateable hydroxyl group, the ester acetate showing no OH absorption in the I. R., and, since the ester itself possesses a single sharp band in the carbonyl region ($\lambda_{max}^{CH_2Cl_2}$ 5·77 μ), it is presumed that the fourth oxygen is present in an ether group. The microanalytical results on ester, ester acetate, and diol fit the formula $C_{32}H_{52}O_4$ for the ester. The diol was found to contain a methoxyl group [found $(O)CH_3$ 3·23%, $C_{31}H_{52}O_3$ requires 3·18% for one $(O)CH_3$] so that the

Table I. Methyl Esters

	Formula	Found		Calculated		m. p.* °C	$[\alpha]_D$ /$CHCl_3$	% (approx.)
		C	H	C	H			
A	$C_{32}H_{52}O_4$	76·79	10·52	76·75	10·47	200–201	+77°	0–1·0
B	$C_{31}H_{50}O_3$	78·93	10·60	79·10	10·71	239–241	+39°	1·0
C	$C_{31}H_{50}O_4$	76·15	10·54	76·50	10·36	239–242	+84°	together
D	$C_{31}H_{50}O_4$	76·28	10·24	76·50	10·36	268–271	+76°	50·0
E	$C_{31}H_{50}O_5$	74·18	10·04	74·06	10·03	293–295	+72° (dioxan)	20·0

* All m.p.s. are corrected.

Table II. Methyl Ester Acetates

	Formula	Found		Calculated		m. p.* °C	$[\alpha]_D$ /$CHCl_3$
		C	H	C	H		
A	$C_{34}H_{54}O_5$	74·87	9·85	75·23	10·03	175–176	+34°
B	$C_{33}H_{52}O_4$	77·01	10·40	77·29	10·22	236–237	+39°
C	$C_{35}H_{54}O_6$	73·55	9·47	73·64	9·54	189–190	+46°
D	$C_{35}H_{54}O_6$	73·85	9·61	73·64	9·54	170–171	+49°
E	$C_{35}H_{54}O_7$	71·75	9·45	71·64	9·28	215–216	+62°

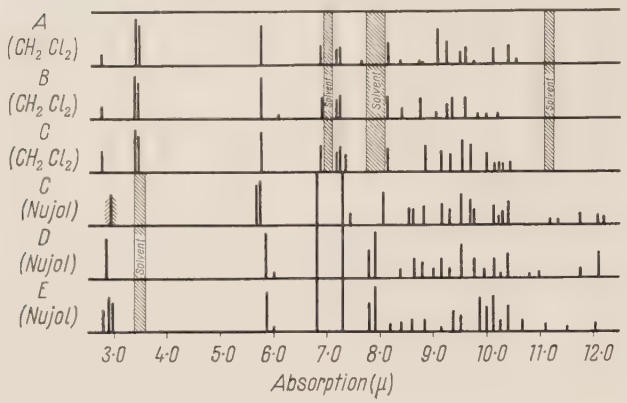
* All m.p.s. are corrected.

Table III. Commols

	Formula	Found		Calculated		m. p.* °C	$[\alpha]_D$
		C	H	C	H		
A	$C_{31}H_{52}O_3$	79·03	10·92	78·76	11·09	294–298	+76° (tetrahydrofuran)
B	$C_{30}H_{50}O_2$	81·39	11·32	81·39	11·38	230–232	+38° (chloroform)
C	$C_{30}H_{50}O_3$	78·43	10·81	78·55	10·99	258–259	+81° (dioxan)
D	$C_{30}H_{50}O_3$	78·63	11·06	78·55	10·99	246–247	+79° (chloroform)
E	$C_{30}H_{50}O_4$	75·79	10·78	75·90	10·62	245–246	+63° (ethanol)

* All m.p.s. are corrected

Table IV. I. R. Spectra of Esters



commic acid-A skeleton calls for C_{30} and 5 rings with one double bond. Methyl commate-B contains one acetylateable hydroxyl group. Methyl commate-C and -D each contain two acetylateable hydroxyl groups, as does methyl commate-E, but the latter has an additional hindered hydroxyl group that appears in the I. R. spectrum of the diacetate ($\lambda_{max}^{CH_2Cl_2}$ 2·78 μ).

Further work on the chemistry of these substances will be reported elsewhere.

A. F. Thomas* and J. M. Müller

Forschungslaboratorien der CIBA Aktiengesellschaft, Pharmazeutische Abteilung, Basel, November 2, 1959.

Zusammenfassung

Die Isolierung von fünf neuen Triterpensäuren («Commisäure-A bis -E», englisch «commic acid-A to -E») als Methylester aus *Commiphora glandulosa* Harz wird beschrieben. Sie wurden als Acetate bzw. als die entsprechenden Alkohole charakterisiert.

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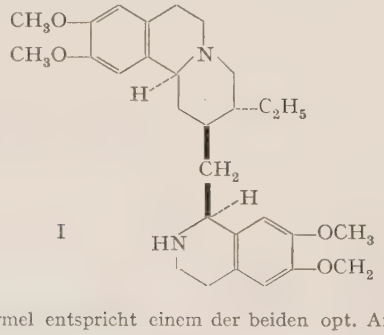
Stereospezifität der Wirkung von Emetin

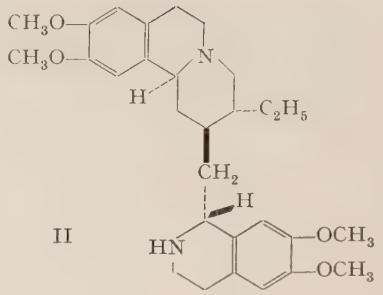
Die amöbicide Wirkung und Toxizität von totalsynthetisch hergestelltem rac. Emetin, rac. Isometin (I)[1] und der beiden optischen Antipoden (−)-Emetin (II) und (+)-Emetin (Spiegelbild von II)[2] wurden *in vitro* und *in vivo* bestimmt und mit natürlichem Emetin[3] verglichen. Sämtliche Präparate wurden als Dihydrochlorid-hydrate geprüft.



[1] Die Formel entspricht einem der beiden opt. Antipoden des Racemates.

[2] A. Brossi, M. Baumann und O. Schnider, Helv. chim. Acta *42*, 1516 (1959).

[3] Emetin-hydrochlorid, Ph. H. V. (Sandoz AG., Basel).



Technik. Unsere Versuche wurden mit *Entamoeba histolytica*, Stamm Moore, gehalten auf einem diphasischen Nährboden, bestehend aus Eikoagulat und Stones-Locke-Lösung, zusätzlich einer Polybakterien-Flora, durchgeführt. Die Aktivität *in vitro* wurde im Shaffer-Frye-Medium bestimmt und entspricht der minimalen Präparate-Konzentration, bei der mikroskopisch nach 24stündiger Bebrütung in der Nährflüssigkeit keine lebenden Amöben mehr gefunden wurden. Die Aktivität *in vivo* wurde nach der Technik von Jones (modifiziert) bestimmt und entspricht der Menge des einmalig oral gegebenen Präparates in mg/kg, die 6 Tage nach Verabreichung bei 50% der Ratten zum Verschwinden des Erregers führt. Die Toxizität wurde an der Ratte nach der Probitmethode, bei Verwendung von 4 Tieren pro Dosis und einer Beobachtungszeit von 10 Tagen bestimmt.

Resultate

Präparat	Toxizität	Aktivität *in vitro*	Aktivität *in vivo*
	DL_{50} Ratte s. c. mg/kg	Endwert in γ/ml	CD_{50} Ratte oral mg/kg
Nat. Emetin	25	10	9,5
(−)-Emetin (II) . . .	17	10	6,0
(+)-Emetin*	700	1000	170,0
Rac. Emetin	35	20	13,0
Rac. Isoemetin (I)[1] . .	1400	1000	200,0

Diskussion. Aus diesen Untersuchungen geht hervor, dass totalsynthetisch hergestelltes (−)-Emetin (II) die gleiche akute Toxizität und amöbicide Wirkung aufweist wie das natürliche Alkaloid. Das unnatürliche (+)-Emetin ist hingegen unwirksam, aber auch wenig toxisch. Dementsprechend ist rac. Emetin etwas weniger toxisch und weniger wirksam als (−)-Emetin (II). Das rac. Isoemetin (I)[1] ist wenig toxisch, aber auch unwirksam, was für seinen (−)-Antipoden in orientierenden klinischen Versuchen bestätigt wurde[4]. Demnach ist die amöbicide Wirkung von Verbindungen aus der Emetinreihe konfigurationsspezifisch.

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*Forschungsabteilung der F. Hoffmann-La Roche & Co., A.G., Basel und * Instituto Nacional de Endemias Rurais, Belo Horizonte (Brasilien), 8. September 1959.*

[4] T. A. Henry, *The Plant Alkaloids* 4th ed. (J. & A. Churchill, London 1949), p. 402.

Summary

(−)-Emetine, as prepared by total synthesis, has the same antiamebic activity and toxicity as the natural alkaloid. On the contrary its antipode and rac. isoemetine are not active and practically non-toxic. A relationship between configuration and antiamebic activity has thus been demonstrated for various representatives of the emetine series.

Genetisch bedingte Tumoren und der Gehalt an freien Aminosäuren bei *Nicotiana*[1]

Seit den Untersuchungen von KOSTOFF[2] kennen wir viele Artbastarde von *Nicotiana*, die «spontan», das heisst ohne nachweisbare äussere Reize, allein auf Grund der genetischen Konstitution Tumoren bilden[2–5]. Die stofflichen Ursachen dieser Tumorbildung sind noch unbekannt[5].

Frühere Untersuchungen über cecidogene Agenzien lenkten nun unsere Aufmerksamkeit auf die Stoffgruppe der Aminosäuren, weil mehrere dieser Substanzen in hoher Konzentration und in bestimmten, aber sehr mannigfaltigen Kombinationen am Pflanzenkörper Gewebswucherungen verursachen können. Voraussetzung hierfür ist, dass das Pflanzengewebe eine hohe Mitoseaktivität besitzt. Die Wirkung dieser Substanzen ist unspezifisch[6].

Um zu prüfen, ob womöglich auch die genetisch bedingten Tumoren beim Tabak durch solche Substanzen, die in diesem Falle vom Genotyp der Pflanze bereitgestellt werden müssten, induziert werden, haben wir als erstes den Gehalt an freien Aminosäuren bei jungen, physiologisch vergleichbaren und gesunden Pflanzen bzw. Pflanzenorganen von *Nicotiana glauca* und *N. langsdorffii*, die stets tumorfrei sind, und einem amphidiploiden Bastard dieser beiden Arten, der zur Blütezeit spontan Tumoren bildet, bestimmt und verglichen[7]. Die hier vorliegenden Untersuchungen unterscheiden sich von fast allen uns bisher bekanntgewordenen entsprechenden Arbeiten zum Tumorproblem dadurch, dass wir nicht den Tumor selbst, sondern das noch gesunde, aber durch die Bastardierung zur Tumorbildung erblich disponierte Gewebe analysieren und mit demjenigen der stets tumorfreien Ausgangsgenotypen vergleichen.

In Abbildung 1 ist das Summenergebnis der Extinktion (Zeiss, Spektralphotometer mit Chromatogrammansatz; 450 mμ) aller proteinfreien ninhydrinpositiven Substanzen aus Blatt- und Sprossgewebe dargestellt. Danach verhalten sich die Extinktionswerte von *N. glauca: N. langsdorffii:* Bastard *N. glauca/langsdorffii*) wie 1:1,24: 1,62. Der Gehalt an proteinfreien ninhydrinpositiven Körpern ist also beim Artbastard wesentlich höher als bei den Ausgangsformen.

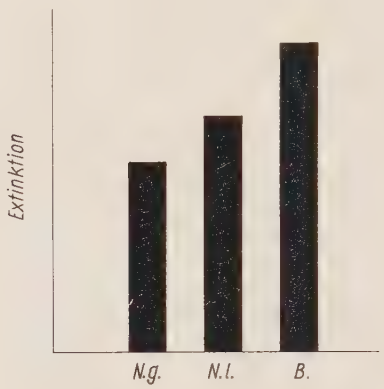

Abb. 1. Summenergebnis der Extinktion (*N. g.* = *Nicotiana glauca; N. l.* = *N. langsdorffii; B.* = amphidiploider Bastard *N. glauca/langsdorffii*). Einzelheiten im Text

Die weitere Analyse des Gehalts an freien Aminosäuren erbrachte das in Abbildung 2 dargestellte Ergebnis. Hier

[1] Mit Unterstützung durch die Deutsche Forschungsgemeinschaft.
[2] D. KOSTOFF, Z. Bakteriol. Parasitenk. Abt. II, *81*, 244 (1930).
[3] A. E. KEHR und H. H. SMITH, Brookhaven Symp. Biol., No. 6, 55 (1954).
[4] A. C. BRAUN und T. STONIER, Protoplasmatologia *10*, 1 (1958).
[5] H. H. SMITH, Ann. N. Y. Acad. Sci. *71*, 1163 (1958).
[6] F. ANDERS, Exper. *14*, 62 (1958); Verh. dtsch. Zool. Ges. in Frankfurt a. M. *1958*, 355; Biol. Zbl., im Druck.

[7] Wir danken Herrn Prof. Dr. A. C. BRAUN, Rockefeller Institute for Medical Research, New York, und Herrn Prof. Dr. K. SCHMID, Bundesanstalt für Tabakforschung, Forchheim bei Karlsruhe, für die bereitwillige Überlassung von Tabaksamen.

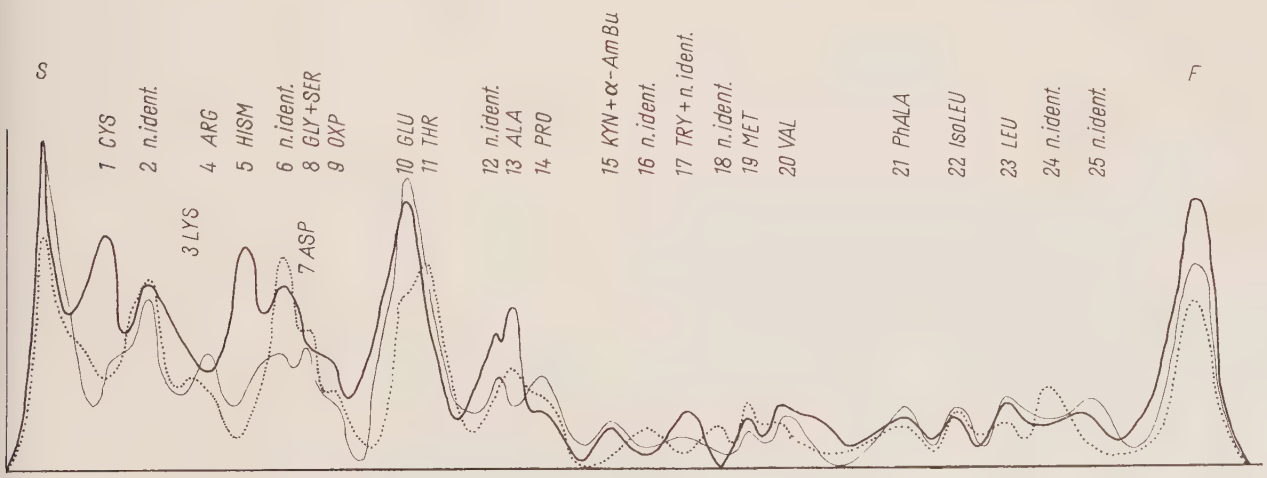

Abb. 2. Extinktionskurven von Papierchromatogrammen von *N. glauca* (punktiert), *N. langsdorffii* (dünn ausgezogen) und dem amphidiploiden Bastard *N. glauca/langsdorffii* (*dick* ausgezogen). *S* = Startpunkt; *F* = Steigfront. Einzelheiten im Text

sind die Extinktionskurven von je drei Papierchromatogrammen von Aminosäureextrakten dargestellt (Extrakte aus je 500 mg Tabak mit H_2O und $n/100$ HCl; Abtrennung der Proteine mit Trichloressigsäure; schonendes Einengen auf 0,5 ml; Auftragen aliquoter Mengen und ausgewählter Testgemische auf S. u. S. 2043b mgl; entwickelt wurde aufsteigend mit Butanol-Eisessig-Wasser 4:1:1). Die im Kurvenbild zu erkennenden 25 Maxima entsprechen den in der Tabelle aufgeführten 28 Substanzen, von denen bisher 20 identifiziert werden konnten. Es zeigt sich, dass der Bastard wesentlich mehr freies Cystin (Nr. 1), Histamin (Nr. 5), Oxyprolin (Nr. 9), eine bisher nicht identifizierte ninhydrinpositive Verbindung (Nr. 12), Alanin (Nr. 13), Kynurenin (Nr. 15) und Tryptophan (Nr. 17)[8] enthält als die Ausgangsarten. Die übrigen Substanzkonzentrationen schliessen sich zumeist denjenigen von *N. glauca* oder *N. langsdorffii* an oder liegen zwischen beiden; nur in Ausnahmefällen – wie bei Substanz Nr. 18 – liegen sie tiefer. Der Summenvergleich der addierten γ-Werte in der Tabelle schliesst sich mit 1:1,07:1,48 zwangslos dem Summenergebnis der Extinktion (Abb. 1) an.

Es hat sich also gezeigt, dass die Menge aller ninhydrinpositiven Substanzen beim Bastard wesentlich höher ist als bei den Ausgangsarten und dass der Bastard wenigstens 6 dieser Stoffe in einer auffallend hohen Konzentration enthält. Dieses Ergebnis mag zunächst überraschen; es stellt jedoch keine Besonderheit dar, wenn man die starke Anreicherung von freien Aminosäuren im Bastard als Heterosiseffekt auffasst. In welchem Masse die besonderen chromosomalen Verhältnisse der Amphidiploidie an der Erhöhung des Aminosäurespiegels beteiligt sind, bedarf der weiteren Nachprüfung.

Tabelle
Gehalt an freien Aminosäuren in 45 mg Tabak
(γ-Werte, berechnet aus der Extinktion)

Laufende Nr. der Maxima	Aminosäure	*N. glauca*	*N. langsdorffii*	Bastard
1	Cystin	(3,8)*	(4,5)*	9,0
2	nicht identifiziert	5,0 **	4,5 **	4,5 **
3	Lysin	3,0	(3,0)*	(3,8)*
4	Arginin	(2,5)*	4,0	—
5	Histamin	—	—	8,0
6	nicht identifiziert	9,5 ***	5,3 ***	8,1 ***
7	Asparaginsäure	(3,4)*	3,0	(3,8)*
8	Glycin	4 5	—	3,7
	Serin	(4,5)*	(3,0)*	(3,17)*
9	Oxyprolin	2.5	(2,0)*	4,0
10	Glutaminsäure	6,0	10,5	10,5
11	Threonin	6,5	—	—
12	nicht identifiziert	—	2,3 **	3,3 **
13	Alanin	2,5	(1,5)*	4,0
14	Prolin	(6,0)*	10,0	6,0
15	Kynurenin	—	—	1,8
	α-Aminobutters.	—	2,0	—
16	nicht identifiziert	1,0 **	—	—
17	Tryptophan	—	—	4,0
	nicht identifiziert	—	2,0 **	
18	nicht identifiziert	1,0 **	—	—
19	Methionin	3,0	2,0	2,5
20	Valin	1,5	2,0	2,5
21	Phenylalanin	2,0	3,0	2,5
22	Isoleucin	1,8	2,0	1,7
23	Leucin	1,5	2,5	2,2
24	nicht identifiziert	2,5 **	—	—
25	nicht identifiziert	—	2,3 **	1,5 **
	Summenvergleich	53,8 =1,00	57,4 1,07	79,8 1,48

* Hypothetische Werte, da keine Maxima ausgeprägt.
** Berechnet auf Basis der Lysinabsorption.
*** Berechnet auf Basis der Asparaginsäureabsorption.

Es liegt nun nahe, das vorliegende Ergebnis mit demjenigen der eingangs erwähnten Analysen cecidogener Substanzen[6] zu vergleichen. Bei der Untersuchung des Sekrets bzw. Exkrets, mit dessen Hilfe die Reblaus (*Phylloxera*) an der Rebe (*Vitis*) einfach gebaute Beutel- und Krebsgallen induziert, hatte es sich ergeben, dass die wirksamen Bestandteile im wesentlichen mit einem konzentrierten Gemisch aus den Aminosäuren Histidin, Lysin, Tryptophan, Glutaminsäure und Valin (die Identifizierung der beiden zuletzt genannten Substanzen war damals nicht ganz sicher) identisch sind. Diese Substanzen wirken synergistisch, wobei Tryptophan die weitaus stärkste Wirkungskomponente darstellt; die Wirkung der übrigen Substanzen beruht im wesentlichen nur auf einer Verstärkung des cecidogenen Tryptophaneffektes. Bei dem an *Nicotiana* gewonnenen Ergebnis ist nun auffällig, dass Tryptophan nur beim tumordisponierten Bastard in hoher Konzentration vorkommt. Bei *N. glauca* und *N. langsdorffii* konnte diese Aminosäure bisher nicht nachgewiesen werden. Zwar findet sich bei *N. langsdorffii* eine Verbindung, deren R_f-Wert mit demjenigen von Tryptophan übereinstimmt; doch zeigt diese Substanz nicht die für Tryptophan charakteristische Färbung. Ausserdem verlief der Tryptophannachweis nach Winkler und Petersen[9] nur beim Bastard eindeutig positiv. (Im Kurvenbild kommt der hohe Tryptophangehalt nicht genügend deutlich zum Ausdruck, weil der entsprechende Ninhydrinkomplex nur schwach absorbiert.) Auch die beiden cecidogenen Aminosäuren Valin und Glutaminsäure sind in diesem Zusammenhang zu nennen; erstere Substanz tritt nur beim Bastard in relativ hoher Konzentration auf, letztere allerdings auch bei *N. langsdorffii*.

Es ist also unverkennbar, dass in der Stoffzusammensetzung der früher analysierten cecidogenen Agenzien eines Galleninsekts und den beim tumordisponierten Tabakbastard in hoher Konzentration vorliegenden Aminosäuren gewisse Parallelen vorhanden sind. Wir fühlen uns deshalb in der Vermutung sehr bestärkt, dass auch die erblich bedingten Tumoren bei *Nicotiana* im wesentlichen durch Aminosäuren induziert werden.

Möglicherweise liegt hier ein allgemeineres Prinzip einer endogenen, das heisst vom Genotyp selbst verursachten Tumorinduktion vor; denn nach Untersuchungen über die erbliche Tumordisposition bei Art- bzw. Gattungsbastarden von gewissen Zahnkarpfen (Poeciliidae), die bei uns zur Zeit im Gange sind, ist das Auftreten von Tumoren offenbar ebenfalls mit einem unterschiedlichen Vorkommen bestimmter Aminosäuren korreliert.

Eine ausführliche Publikation folgt a. a. O.[10].

F. Anders und F. Vester

Zoologisches Institut und Institut für Organische Chemie der Universität des Saarlandes, Saarbrücken, 12. Oktober 1959.

[8] Der erhöhte Gehalt an freiem Tryptophan wurde schon von J. H. M. Henderson in Zusammenarbeit mit A. E. Kehr und H. H. Smith in Gewebekulturen beobachtet (zit. nach [4]).

[9] S. Winkler und S. Petersen, Hoppe-Seylers Z. *231*, 210 (1935). – S. Winkler, Z. anal. Chem. *105*, 303 (1936).

[10] F. Vester und F. Anders, Z. Biochem., im Druck.

Summary

The amphidiploid hybrid of *Nicotiana glauca* × *N. langsdorffi*, producing tumors spontaneously, contains essentially more of the following free amino acids than the inital species: cystine, histamine, oxyproline, alanine, kynurenine, tryptophane, and a non-identified ninhydrine positive substance. It is supposed that the abnormally high content of free amino acids is connected with the spontaneous formation of tumors.

Genetic and Autoradiographic Studies of Tritiated Thymidine in Testes of *Drosophila melanogaster*[1]

A study on the influence of mating intensity and rate of sperm utilization upon the rate of spermatogenesis in *Drosophila melanogaster* is presently under way. Tritiated thymidine (T–H^3), a specific label for DNA in most biological systems studied (Brachet[2]), is being used in conjunction with autoradiography to label those premeiotic cells which are synthesizing DNA and to follow the development of these cells to mature sperm. In addition, it is planned to use the incorporability of T–H^3 as an indication of the rate of entrance of cells into the premeiotic DNA synthesizing stages. This preliminary report contains information obtained in the earliest of these experiments.

Additionally, in order to gain some insight into the degree of genetic damage caused by the amounts of isotope which must be incorporated to give satisfactory autoradiographs of testicular cells, studies on the rate of production of sex-linked recessive mutations by this compound have been initiated and preliminary data on this subject, also, are herein reported.

To decrease the amount of non-radioactive nucleic acid precursors ingested so that strong autoradiographs could be obtained after a minimum feeding time, application of the isotope was not carried out in the standard fly culture medium which is rich in yeast. Instead, the isotope-containing medium was made up as follows: 0·1 ml T–H^3 solution (Schwarz Laboratories, Sp. Act. 1·9 c/*mM*) with a total activity of 100 microcuries, 0·5 ml of a 1% agar solution and 0·5 ml of a modified Eagle's tissue culture medium.

Twenty-five 16-h Canton-S larvae not identified as to sex were placed upon the above medium for a period of 4 h and then removed to standard corn meal, yeast, and sugar food. Eight of the 25 larvae died during these 4 h, while only two of 25 control larvae died. Two larval males were fixed immediately after the feeding period; three males were fixed 19 h later (39-h larvae) and the remaining four males were fixed 56 h after removal from the radioactive food (76-h larvae). All larvae were fixed in hot 45% acetic acid and imbedded in parlodion and paraffin according to the Peterfis double imbedding technique. Sections were cut at 6 μ and mounted serially. After dissolution of the paraffin and parlodion, and hydration of the sections by means of a graded ethanol series, the slides were treated with 5% trichloroacetic acid at 3–5°C for 5 min to remove any acid soluble isotope. From this point on, autoradiographs were prepared according to the stripping film technique as published by Taylor[3].

At 16 h, the larval testes are composed entirely of spermatogonia with no evidence of any anterior-posterior developmental gradient, except for the difference in size between the apical cells and those slightly larger spermatogonia that have been proliferated from them. The testes of male larvae that were fixed immediately after the 4-h period show activity over the apical region where mitotic proliferation occurs. Also, there are some radioactive nuclei located posteriorly, indicating that very early in spermatocyte development, before the long growth stage, during which the volumes of the nucleus and cytoplasm of primary spermatocytes increase so markedly, the chromosomes are reduplicated in preparation for the meiotic divisions.

19 h following feeding (39-h larvae), there is still some activity present anteriorly but many labeled cells have moved posteriorly (Fig. 1). All nuclei of a cyst are either entirely radioactive, or completely lacking in activity, indicating that the cells of any one cyst are physiologically synchronized, at least in terms of incorporation into DNA.

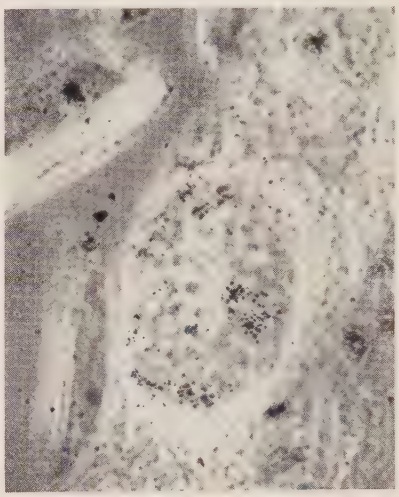

Fig. 1.—39 h larva, 19 h after removal from labeled food. × 850.

56 h after removal from radioactive food (76-h larvae), radioactive primary spermatocytes may be observed in the posterior third of the gonad (Fig. 2 and 3). The radioactivity observed anteriorly derives from interstitial cells located throughout the testis.

While additional work must be carried out to establish the developmental pattern of the testis in relation to the time of DNA synthesis, it may be seen from our preliminary data that spermatocytes reduplicate their chromosomes very early during their life history and move posteriorly as additional cells are proliferated from the apical spermatogonia.

For the measurement of the mutagenic effect of T–H^3, the same sample of food used in the previous experiment was mixed with 1·1 ml of the standard fruit fly medium: agar, 0·8%; sugar, 5%; corn meal, 6%; yeast, 1·5%; water, 86%.

[1] Supported in part by Grant G-6097 of the National Science Foundation.

[2] J. Brachet, Exp. Cell Res. Suppl. *6*, 78 (1959).

[3] J. H. Taylor in *Physical Techniques in Biological Research*, Vol. 3 (Ed. G. Oster and A. W. Pollister, Academic Press, N. Y. 1956), p. 545.

Fig. 2.—76-h larva, 56 h after removal from food. Radioactivity of nuclei of one cyst illustrated. × 850

16 h after hatching, 50 Canton-S larvae were placed on this food and, as pupae appeared, they were removed and placed in a vial with standard food. Although the 16-h larvae, which were placed upon the radioactive food, hatched from eggs that were laid over a 2-h period, there was a spread of two days in pupation time. Furthermore, there was a spread of four days in the eclosion of adult males from these pupae. In the case of a group of 50 control larvae, pupation occurred over a period of two days and the eclosion of adults from these pupae over a period of three days.

Accordingly, the following groups were derived:

Group	*Days after pupation*	*Number of ♂♂*
I	5	6
II	6	5
III	6	5
IV	7 and 8	5

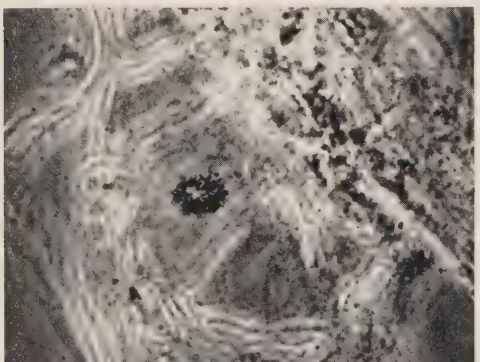

Fig. 3.—Same larva as in Figure 2. Heavily labeled nucleus of posterior spermatocyte illustrated. The large size of cells and nuclei at this time prevent the visualization of labeled nuclei of any one cyst in a single section. × 850

On the day following their appearance, the adult males were mated to Muller-5 females for the purpose of determining the sex-linked recessive mutation rate. The males of Groups I, III, and IV were mated individually to three females, each for a period of three days, and were then provided with a second group of three females each and so on, for a total of four broods. Males of Group II were mated in mass culture of three females per male and four broods obtained.

The Table summarizes the data derived from the four separate groups. Unquestionably, the presence of the tritium produces a mutagenic effect. With the exception of Group II, the numbers are generally small and the attendant sampling error correspondingly large, so that a detailed analysis is unwarranted. However, it may be seen that in all cases the highest mutation rate is present in the second brood.

Table

	Brood	Mating period	Non-lethal chromosomes	Lethal chromosomes	% lethal chromosomes
Group I . . .	1	1–4 days	126	1	0·78
	2	4–6 days	440	6	1·35
	3	6–9 days	81	1	1·22
	4	9–12 days	120	1	0·82
Group II . .	1	1–3 days	226	2	0·88
	2	3–6 days	448	18	3·90
	3	6–9 days	365	2	0·54
	4	9–12 days	465	3	0·64
Group III . .	1	1–3 days	175	4	2·23
	2	3–6 days	78	3	3·70
	3	6–9 days	47	1	2·08
	4	9–12 days	50	0	—
Group IV . .	1	1–3 days	102	2	1·92
	2	3–6 days	52	2	3·70
	3	6–7 days	34	0	—
	4	7–12 days	87	3	3·33
Controls . . . (mass matings)	1	1–3 days	1,701	1	0·06
	2	3–6 days	1,646	4	0·24
	3	6–9 days	935	0	—

It is still to be determined whether this is a case of differential sensitivity, or merely whether the population of labeled sperm has been diluted by unlabeled sperm which had reduplicated their chromosomes, either before the T–H³ was presented or after the labeled pool had been depleted. The question of clusters of mutations also remains unresolved. However, the observed mutations are well distributed among the individual males whose progenies have been tested. For example, in Group I, Brood 2, the six mutations occur in four different males, two of which carried two mutations each. Furthermore, where more than one mutation has been scored for any single brood, the mutant chromosomes have not been restricted to any individual male.

Plaut[4] discusses the question concerning the possible biological damage to chromosomes that have incorporated T–H³ and points out the need for experimental work to

[4] W. Plaut, Labor. Invest. *8*, 286 (1959).

examine this question. PAINTER, DREW, and HILL[5], TAYLOR[6] and WIMBER[7] all present data which indicate that endogenous radiation can cause damage to the chromosomes. This brief report indicates the possible intrachromosomal damage that may follow the incorporation of radioactive compound into the chromosomes.

Larger scale experiments in which precise quantities of T–H^3 are injected into larvae will be carried out to examine more critically the mutagenic properties of the tritium atom after its incorporation into the chromosomes.

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Departments of Genetics and Experimental Pathology, City of Hope Medical Center, Duarte (Calif.), August 20, 1959.

Zusammenfassung

Die Aufnahme von H^3-Thymidin in 16 h alte männliche Larven von *Drosophila melanogaster* wurde autoradiographisch verfolgt und die mutagene Wirkung des einverleibten H^3-Thymidin durch die Bestimmung der Häufigkeit der geschlechtsgebundenen rezessiven Mutationen gemessen. Es zeigte sich, dass das der männlichen Larve der *Drosophila melanogaster* verabreichte H^3-Thymidin eine bedeutende Häufigkeit der Mutationen verursacht.

[5] R. B. PAINTER, R. M. DREW, and W. L. HILL, Science *127*, 1244 (1958).
[6] J. H. TAYLOR, Genetics *43*, 515 (1958).
[7] D. W. WIMBER, Proc. Nat. Acad. Sci., Wash. *45*, 839 (1959).

Hormonal Control of Mating Behavior in an Insect[1]

Mating behavior in insects was not demonstrated until very recently to be influenced by endocrine glands. At one time it was thought that the activity of the gonads might be involved in this behavior, but it was shown that, for example, in *Lymantria*[2], *Gryllus*[3], and *Leucophaea*[4] gonadectomy in males as well as in females does not impair mating activity. Confirmatory results are also given here (Table). Furthermore, in females of *Diploptera*[5,6] mating occurs shortly after emergence, when the gonads are still inactive. Recently, it was observed that after the implantation of nymphal prothoracic glands into adult females of *Leucophaea* only a small percentage of these females mated[7]. This experiment was repeated, and again only 4 of 16 (25%) experimental animals accepted a male within 4 weeks after emergence. Apparently, the implantation of active prothoracic glands changes the normal response of adult females to courting males.

It remained to be seen whether this effect was direct or indirect, since implants of active prothoracic glands in adult females of *Leucophaea* inhibit the activity of the corpora allata. To test this point, females of *Leucophaea* were allatectomized one day after emergence and then were kept with normal males. In the experimental as well as control series 2 couples were placed together in each finger bowl, so that each female had a chance to mate with either of the 2 males.

In the normal control series, 90% of the females had accepted a male within 26 days after emergence as seen by the presence of a spermatophore in the bursa copulatrix (Table). By contrast, only about 30% of the allatectomized females accepted a male within the same period. Sham operated females mated as readily as normal animals (Table). From this it seems that mating in females of *Leucophaea* depends on the presence and activity of the corpora allata. This conclusion is supported by the observation that, in *Leucophaea*, mating normally occurs at a time when the corpora allata show histological signs of beginning activity, i. e. an increase in cytoplasmic content. Thus, the effect of the implantation of active prothoracic glands on the female response to courting males can be accounted for by the inhibition of the corpora allata[7].

Mating in normal and experimentally treated *Leucophaea*

	No. of animals observed	No. of animals mated	
		within 26 days after emergence	within 12 days after re-impl. of corpora allata
1. Normal females	156	141 = 90 %	—
2. Castrated females	16	15 = 94 %	—
3. Normal females mated with castrated males	58	55 = 95 %	—
4. Allatectomized females	36	11 = 30·5 %	—
5. Sham allatectomized females	16	14 = 87 %	—
6. Allatectomized females that received four active corpora allata 26–30 days after emergence	11	—	9 = 82%

This conclusion is further substantiated by the following experiment: Eleven allatectomized females were kept with normal males for 26–30 days during which period they did not mate. Each of these 11 females then received implants of 4 active corpora allata taken from last instar nymphs 3–5 days after their molt. Corpora allata of such donors are known to be active, since after implantation into adult females they bring about egg maturation. Five days after the implantation of the corpora allata, 6 females had already accepted a male, and on the 12th day after the implantation a total of 9 (82%) of the experimental animals had mated (Table). In the 2 animals that had not mated when the experiment was discontinued on the twentieth day after re-implantation, the implanted corpora allata must have been quite inactive because the oocytes of these animals contained very little yolk. This experiment with re-implantation of the corpora allata demonstrates that in *Leucophaea* active corpora allata are involved in the responsiveness of females to courting males.

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[7] F. ENGELMANN, Z. vgl. Physiol. *41*, 456 (1959).

What is the possible mechanism of this hormonal effect on mating behavior in an insect? As Roth and Willis[8] have shown, the female of some cockroach species, including *Leucophaea*, takes an active part in courtship. An odorous substance excreted by the male causes the female to feed on his tergal gland, and by so doing she stimulates the male to copulate. Unless the female responds in this particular way to the courting male, no mating occurs. To test whether the antennae serve as the organs of smell in this female behavior, the antennae were removed from 16 females shortly after emergence. None accepted a male in an observation period of 26 days. Perhaps, then, the failure of allatectomized females to mate depends on some alteration of their ability to perceive the male odor. Whether the corpus allatum hormone acts on the olfactory center in the brain or directly on the sensory receptors on the antennae, or whether it conditions the female in an entirely different way cannot be determined at the present time.

It will be of interest to learn whether in other insect species the corpora allata likewise influence the mating behavior of the females. Experiments in this direction are under way. It might be worth mentioning that in *Diploptera* allatectomized females are courted and mate as readily as normal animals. The female of *Diploptera*, however, differs from *Leucophaea* in that she plays a rather passive role during courtship[5]. The female of the *Cecropia* moth is also more or less inactive during courtship; even the isolated abdomen of the female is properly mated by the male[9]. Perhaps the corpus allatum hormone conditions the females only in those species where the female actively takes part in courtship, i. e., in species where the proper sequence of courting events consists in mutual interaction between the sexes.

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Department of Anatomy, Albert Einstein College of Medicine, New York, October 15, 1959.

Zusammenfassung

Von normalen *Leucophaea*-Weibchen werden etwa 90% nach der Adulthäutung innerhalb von 4 Wochen begattet, von allatektomierten Weibchen unter gleichen Zuchtbedingungen nur etwa 30%. Wurden allatektomierten Weibchen 26 Tage post operationem aktive Corpora allata reimplantiert, so kopulierten 82% innerhalb von 12 Tagen. Kastrierte Weibchen zeigten Normalverhalten.

Die Wirkung der Allatektomie auf die Kopulationsbereitschaft wird diskutiert.

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The Intercalated Disc of the Goldfish Heart[1]

Previous electron microscopic investigations have revealed the presence and the fine structure of the intercalated disc in the cardiac muscle of a variety of vertebrates, e.g., mammal[2–6], chick[7], turtle[8], and the toad[9]. The intercalated disc is one of the first structures to arise in the embryonic cardiac tissue[7,10–12]. Its origin and micromorphology are intimately related to those of intercellular bars[8,9,11,12], and its structure is also quite similar to that of the Z-line of cardiac and striated muscle[9,10]. The intercalated disc, in fact, appears to be a specialized type of the ubiquitous intercellular bar, modified in certain contractile tissues such that it interrupts, in simple or complex pattern, the longitudinal myofibrillar pattern, whether found in simple forms as hydroids[13] or in complex forms as in the insectan dorsal vessel[11], and as in the higher vertebrate cardiac muscle, and conducting system[12,14]. It, therefore, would be extremely surprising, and significant, for cellular theories and for phylogenetic concepts, were the intercalated disc to be lacking in the fish as previously reported[15,16].

Electron microscopic preparations were made of the various regions of the heart of the common goldfish (*Carassius auratus*). The methods were the standard ones of fixation in 2% buffered osmium tetroxide, dehydration in graded ethanols, infiltration with a 9:1 mixture of butyl- and methyl-methacrylates, and final embedding in the mixture of methacrylates, initiated with 2% Lucidol, and polymerized at 47°C. Thin sections were cut with a Porter-Blum microtome, using a diamond knife, and the sections examined in a Siemens' Elmiskop I.

Results. – As in mammals, the heart muscle is cellular. The fibers themselves are narrow, one to three fibrils wide in the plane of sectioning (Fig. 1). The cytoplasm appears granular, and contains a large number of mitochondria, which are often long or irregular in shape (Fig. 1). Pinocytotic vesicles are frequently observed, and the endoplasmic reticulum is abundant and highly organized. The Golgi complex is often large, contains the several vesicular and dictyosomal components, is usually located in the residual cytoplasmic area, and often has multivesicular bodies[17] associated with it. The fibrils show all the cross striations including a well-developed N-line (Fig. 7). The Z-band is relatively less dense and less wide than in the mammalian cardiac fiber but shows the vesicular nature (Fig. 2, 4, 7) and has the relations to the endoplasmic reticulum that are common to the mammalian fiber.

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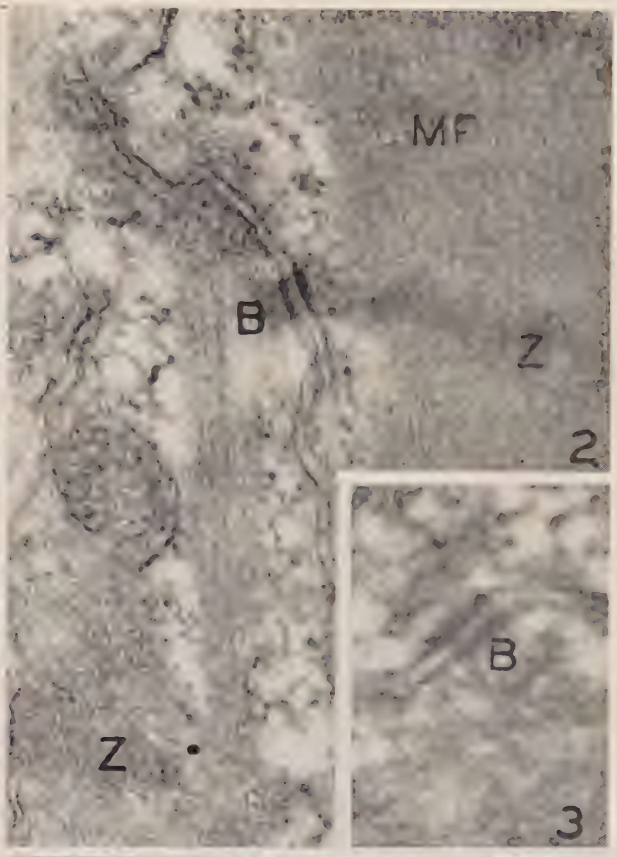
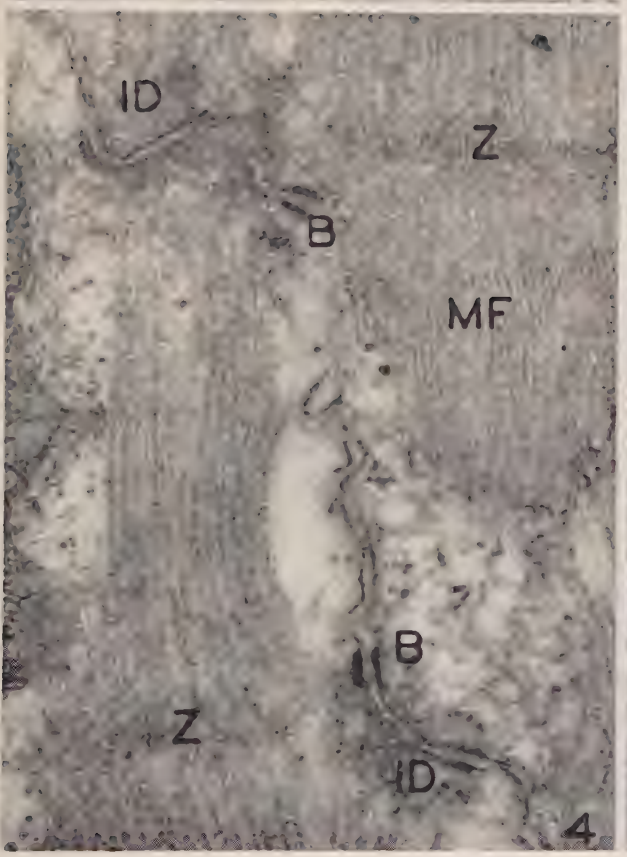




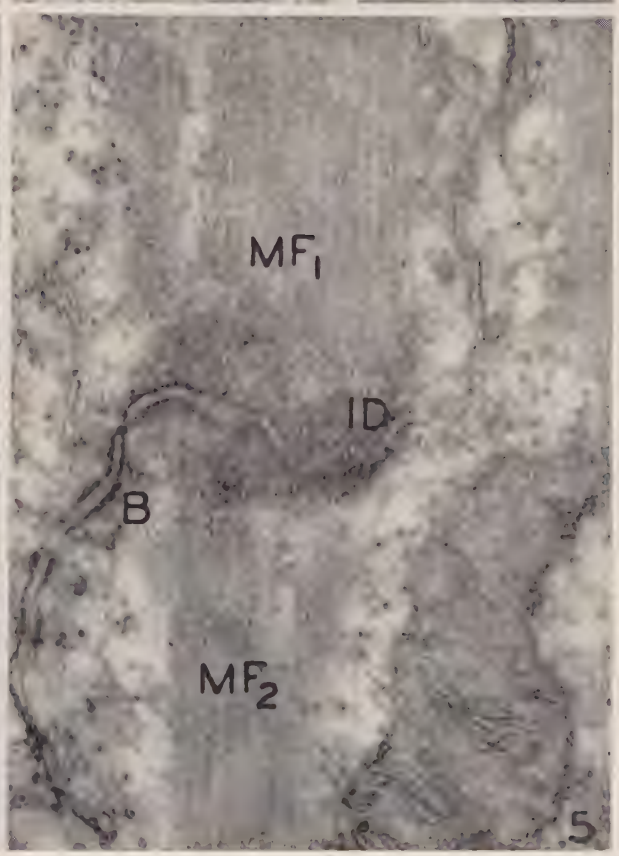


Fig. 1. — Three fibers of the goldfish ventricle. Two of the cells are interrupted by intercalated discs (ID), cells I and II being separated by unmodified plasma membranes and some basement membrane material. The fibers are usually 1–3 myofibrils wide. × 30000

Fig. 2. — Two fibers showing an intercellular bar (B) at the level of the *Z* band of the myofibril (MF) at the right. A specialization of the abutting plasma membranes in the form of a doubling of membranes occurs at the level where the myofilaments terminate just above the bar. × 80000

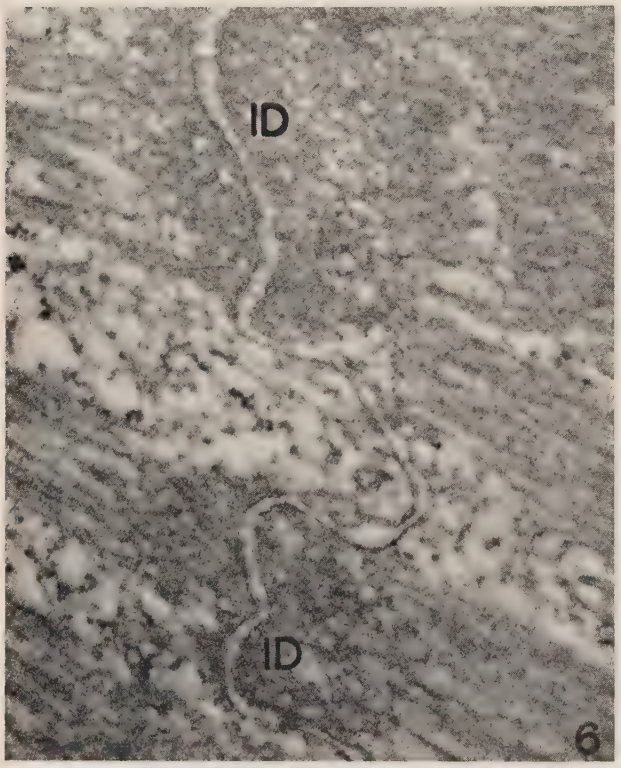
Fig. 3. — Detail of an intercellular bar, showing the oriented intercellular substance between the modified plasma membranes. × 100000

Fig. 4. — Two abutting cells in which the longitudinal myofibril pattern is interrupted by step-wise simple intercalated discs (ID). The cell borders in the interfibrillar regions are specialized as intercellular bars. Both disc and bar substances are continuous with the *Z* substance of the peripheral myofibril. × 70000

Fig. 5. — Simple intercalated disc (ID) interrupting myofibrils (MF1 and MF2) in two abutting ventricular cells. To the left of the disc is an intercellular bar in the non-fibrillar portions of the cell borders. × 70000

Intercellular abutments are of three types, all of which have been observed in higher vertebrate forms. The most common is the simple intercalated disc, interrupting the longitudinal patterns of single myofibrils (Fig. 1, 4, 5). These often occur in stepwise fashion, the steps being one sarcomere long (Fig. 4). Complex discs, interrupting the pattern of more than one fibril (Fig. 6) also occur, although less commonly. These discs are convoluted but not to the same degree as those of the mammalian cardiac muscle. Either type of intercalated disc may at times be continuous with Z band substance (Fig. 4) and indeed the Z band and disc substances appear to be similar. The similarity is not as striking in the fish as in the mammal, however, in that the Z bands are generally of little density, whereas the discs may be of considerable density and indeed of total width up to 1/3 μ. The disc itself comprises modified plasma membranes, vesicles, filaments, a disc substance, and an intercellular cement of medium density. Structures quite similar in their detail, but shorter, not normal to the long axis of the cells, and not always related to contractile filaments, have been observed associated with, but at times distant from, the intercalated discs. These are termed intercellular bars (Fig. 2–5). They seem to be always associated, at least in one of the abutting cells, with the Z band of a fibril. These intercellular bars correspond to, and include, structures previously referred to as mirror plaques[9], desmosomes[8], and specialized regions of the disc[6].

The results of the present study show that an intercalated disc, closely resembling that in mammalian cardiac muscle, exists in the heart of the fish. In addition, more examples of intercellular bars have been found, in close association with the intercalated disc[8], and the Z band, affording further evidence that these three structures are related to one another.



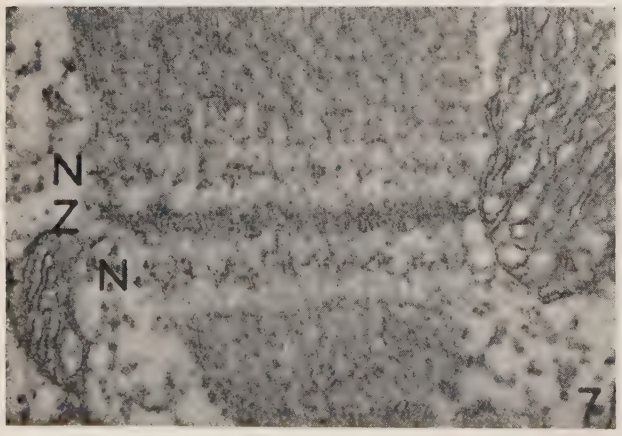


Fig. 6.—Complex intercalated disc (ID) in fish heart, interrupting the longitudinal pattern of several myofibrils. The complex disc of the fish is less convoluted than that of the mammal. × 60000
Fig. 7.—Detail of I region of a myofibril of the ventricular muscle fiber, showing *N* lines on either side of the fairly light *Z* band. × 60000

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Department of Physics, University of Alberta in Calgary (Canada) and Division of Laboratories and Research, New York State Department of Health. Albany, September 4, 1959.

Résumé

L'examen au microscope électronique de coupes minces du cœur de cyprin doré révèle la présence de disques intercalaires de forme soit simple, soit complexe, semblables aux disques des vertébrés supérieurs. De plus, les barres intercellulaires sont présentes. La structure et les rapports des disques et des barres avec la bande Z de la myofibrille suggèrent leur proche similarité.

The Importance of Autoplastically Transplanted Pituitaries for Survival and for Regeneration of Adult *Triturus*

The essential role of the pituitary for the initiation of regenerative processes having been discovered in 1926[1] and definitively established in a series of other researches[2–4], it became imperious to investigate firstly, whether heterotopically transplanted pituitaries were able to support the vital functions indispensable for survival, and secondly, whether grafted pituitaries away from their normal nervous connections, were still capable of providing the required stimuli for regeneration. Autoplastic transpantations were performed on 163 adult *Triturus viridescens* (kept at 20° ± 1° C) into the anterior *eye-chamber*, the *manus* or the *tarsus*. Direct observation under the binocular permitted to establish vascular connections between the eye and the pituitary within 5 to 10 days; for the manus and the tarsus it was established by histological examinations that vascularization occurred somewhat later in most, but not in all the cases.

I. *Survival patterns after autoplastic pituitary transplantations.* Hypophyseoprivic controls begin to die fifteen days after the operation and only 11% live as long as fifty

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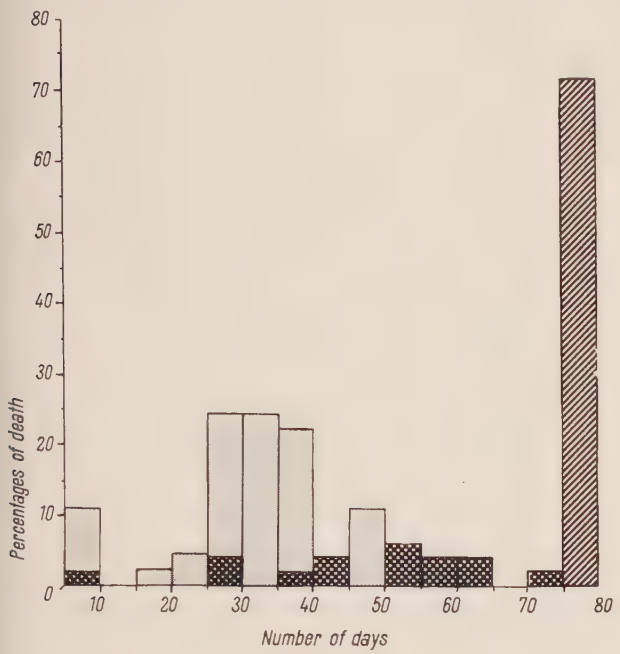
days. Newts with heterotopic transplants shed their epidermis regularly and keep their pigmentation as do normal animals. The data condensed on the histiogram (Fig.) show that hypophysectomized animals may be kept alive for an indefinite length of time; we carry at present numerous cases having survived for over two years.



Histiogram of death patterns of hypophysectomized newts (dotted colums) and of hypophysectomized newts bearing autoplastically transplanted pituitaries (checkerboard columns). The column with diagonal lines on the right represents the percentage of graft-bearing *Triturus* having lived longer than 75 days. (This column, then, refers to survival, rather than to death patterns.) The first double column (from 1 to 10 days) represents early post-operatory deaths within the two experimental series.

II. *The influence of autoplastic pituitary transplants upon forelimb regeneration* may be correlated, particularly in the eye-chamber cases, with their status of vascularization (Table, 3rd horizontal column, but details on vascular connections are not specified): among 47 survivors, 36 had displayed within five to ten days visible vascular loops; subsequently, of these, 34 regenerated normally and 2 did not; conversely, among the 11 cases where no vascularization was observable only 3 did regenerate their limbs. Other differences in regard to regeneration were observed between 'young' and 'old' transplants.

(a) *'Young' transplants* are cases where amputation of the forelimbs was performed simultaneously with the pituitary transplantation. The data of the Table show that 82 (or 80·2%) of these animals exhibited normal regeneration, while 20 (or 19·6%) failed to regenerate. In control experiments with hypophyseoprivic newts 6 (15%) did regenerate and 34 (85%) did not. Histological studies have, in addition, revealed a relation between the site of transplantation and successful regeneration: better 'takes' are obtained from pituitaries placed within the larger tarsus than when transplanted into the much smaller manus of newts; they also showed that, when total resorption of the graft was diagnosed on sections no regeneration took place.

(b) In *'old' transplants* forelimb regeneration was tested 7 to 8 months after transplantation (Table). Results from these animals differed from the previous group since, in spite of healthy appearance and normal pigmentation, 27 among 38 cases showed appreciable delays in the initiation of regenerative processes.

Table

Site of autoplastic transplantations of pituitaries and their effects upon regeneration in forelimbs of *Triturus viridescens*. (A control series dealing with the effects of hypophysectomies and amputation without concomitant heterotopic transplantations is included.)

Site of autoplastic transplantation	Total number of operations	Number of cases observed	Observations at 30 days regeneration		
			Normal	Absent	Delayed
Carpal region of forelimb	35	32	24	8	—
Tarsal region of hind limb	30	23	21	2	—
Anterior eye chamber					
(a) young transplants	48	47	37	10	—
(b) old transplants .	50	38	10	1	27
Hypophysectomy and amputation . . . . (without transplantation)	45	40	6*	34	

* Numerous previous investigations performed at this laboratory had shown, that no matter how carefully performed the hypophysectomies are, there are always some cases (appr. 15%) in which small remnants of pituitaries (discovered upon histological examination of the cranial cavity in supposedly completely hypophysectomized newts) account for regeneration in such cases (Hall and Schotté[3]). This explains the occurrence of positive regeneration in the hypophysectomized newts of the control series.

Conclusions. — These experiments show: that (1) autoplastically transplanted anterior pituitaries may become properly vascularized within a matter of days and that they may remain 'alive' for many months within the eye-chamber and the carpal or tarsal regions; (2) heterotopic pituitaries maintain essential life functions well beyond the normal life expectancy in hypophysectomized newts; (3) after amputation, these transplants are also efficient to support regeneration, but this ability depends on adequate vascular supply and after months of sojourn in heterotopic position a slowing down in the initiatory phases of regenerative processes becomes noticeable. It is therefore concluded that a transplanted pituitary is capable of maintaining in its new site a level of activity sufficient to induce the adrenal end of the pituitary-adrenal axis (shown at this laboratory[5–7], to be essential for regeneration) to react positively to the stimulus of even much delayed amputations, in spite of severance of the normal vascular and nervous connections of the pituitary with the hypothalamus.

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Department of Biology, Amherst College, Amherst (Mass.), July 29, 1959.

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Résumé

Une hypophyse greffée autoplastiquement dans la chambre antérieure de l'œil, dans le *manus* ou dans le *tarsus* s'est avérée capable de remplacer l'hypophyse *in situ* dans son action sur la régénération et la survie chez le Triton, à condition que s'établisse une vascularisation satisfaisante du greffon et que la durée de l'expérience ne dépasse pas plusieurs mois.

Does the Ectodermal Proctodaeum Participate in the Development of the Pronephric Duct in Urodele Amphibia?

In 1926 and 1929, Vogt[1] demonstrated conclusively, by means of the vital staining method, that invagination of mesodermal material in urodele Amphibia continues after the neural groove has appeared and the blastopore has taken on a slit-like form. In 1929, I made the same observation and found that stained material of areas lying in the above-mentioned stage on both sides of the blastopore formed somites of the hindpart of the trunk after invagination, and – in some experiments – appeared as a cord-like structure extending along the ventral edge of the somites in the same part of the body. This 'cord' of cells shining through the ectoderm was seen turning off with its caudal extremity in the direction of the ectodermal proctodaeum (Bijtel[2], 1929, Fig. 6, 1931, Fig. 4). After dissection of the living anaesthetized larvae, this structure was supposed to be the hindpart of the pronephric duct. Thus it would seem that the ectodermal proctodaeum had taken part in the development of the hindpart of the pronephric duct. Histological examination of the stained tissues was not possible at that time, as I did not succeed in preserving the vital stains for that purpose.

Staining experiments of O'Connor[3] in *Amblystoma tigrinum* and *Triton taeniatus*, and of van Geertruyden[4] in *Rana fusca*, have proved that the pronephric duct in larvae of these Amphibia is formed by the independent caudal growth of the pronephric rudiment. The caudal extremity of the developing duct joins with a small diverticulum formed by the ectodermal proctodaeum. By blocking up the way taken by the blastema of the pronephric duct, O'Connor[5] did not usually find a pronephric duct behind the obstructing transplant. In some experiments, however, at the caudal end of the trunk, a small tag of a duct-like structure was attached to the cloaca. This observation suggested the possibility that the cloaca should contribute to the development of the hindmost part of the pronephric duct. In this connection, O'Connor referred to my staining experiments mentioned above.

Results of more recent blocking experiments (Bijtel[6]), like those of similar experiments of Gipouloux[7] however, have shown that the pronephric duct is completely missing only in the case when the obstruction is made in an early stage of development of the pronephric rudiment. If part of the blastema of the pronephric duct has passed the place where the obstacle is implanted, this part continues on its way, joins the small diverticulum pushed forward by the ectodermal proctodaeum and gives rise to the small duct-like structure seen by O'Connor. These experiments prove conclusively that the ectodermal proctodaeum does not contribute to the hindpart of the pronephric duct.

One may ask: what is the significance of the stained cord of cells extending along the ventral edge of the somites in the hindpart of the trunk in my experiments of 1929? In recent experiments, the same effect could be reproduced. Figure 1 gives an example of it. In the larva VK 24 of *Siredon mexicanum*, the surroundings of the ventral half of the blastopore have been stained in the early neural plate stage. In about stage 35 (Harrison), on both sides of the hindpart of the trunk a stained cell cord was seen, extending along the ventral edge of the somites and turning off to the cloaca which was stained as well. As the vital stain could now be preserved, histological examination was possible. It appeared that the stained cord of cells was formed by cell groups of the mesonephros which accompany the unstained pronephric duct, as is shown in Figure 2. In none of these experiments was the pronephric duct stained together with the anlage of the mesonephros.

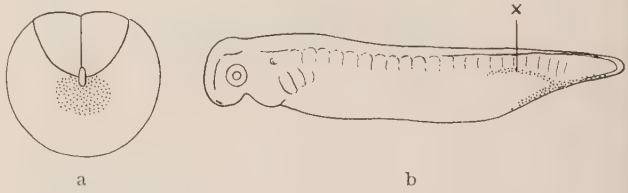

Fig. 1. — Siredon mexicanum VK 24. *a* larva in stage 13 with stained mark round the ventral half of the blastopore. *b* larva in stage 35, *x:* stained mark shining through the ectoderm.

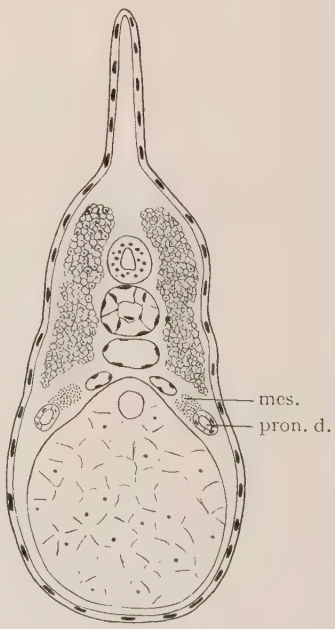

Fig. 2. — Transverse section of larva VK 24 in stage 40. *pron. d.:* pronephric duct; *mes.*: cell group of mesonephros, stained with the vital stain.

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Department of Anatomy and Embryology of the State University of Groningen (Netherlands), August 13, 1959.

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Zusammenfassung

Vom ektodermalen Proctodaeum wächst ein kleiner Divertikel dem Vornierengang entgegen und vereinigt sich mit dessen kaudalem Ende. Einen weiteren Anteil hat das Proctodaeum an der Bildung des Vornierenganges nicht. Zugleich mit dem Proctodaeum invaginiert Material für den Mesonephros, das sich in Zellgruppen entlang dem hinteren Ende des Vornierenganges lagert.

Über eine Strahlenschutzwirkung von Apresolin und C. 5864-Su (2-Octahydro-1-azocynil-äthyl-guanidin)

1957 wurde über die Strahlenschutzwirkung des Rauwolfia-Alkaloids, Reserpin (Serpasil®) berichtet[1]. Diese Schutzwirkung war, gemessen an der Überlebensrate, zwar verhältnismässig gering; sie beansprucht aber Interesse, weil sie mit Dosen erreicht wurde, die erheblich niedriger liegen als etwa bei den Sulfhydrylkörpern Cystein oder Cysteamin[2] und zudem nur einen geringen Bruchteil der akuten Letaldosis darstellen. Ein weiterer wesentlicher Unterschied gegenüber Cysteamin, welches nur schützt, falls es wenige Minuten vor der Bestrahlung verabreicht wird, besteht bei Serpasil darin, dass der Schutzeffekt nach einer Latenzzeit von einigen Stunden manifest wird und sein Optimum zwischen 12 und 24 h nach der Verabreichung erreicht. Wenn man von der absoluten Überlebensrate absieht, ist somit Reserpin von allen bisher bekannten Strahlenschutzmitteln dasjenige mit der längsten Wirkungsdauer. Im Verlaufe weiterer Versuche zur Auffindung von Strahlenschutzsubstanzen mit grosser therapeutischer Breite wurde festgestellt, dass das in der Therapie der Hypertension seit einigen Jahren verwendete Apresolin® (1-Hydrazinophthalazin) sowie C. 5864-Su (2-Octahydro-1-azocinyl-äthyl-guanidin) bereits in verhältnismässig niedriger Dosierung die Maus gegen Bestrahlung mit einer letalen Strahlendosis zu schützen vermögen.

Versuchsbedingungen. Als Versuchstiere dienten männliche Albinomäuse mit einem durchschnittlichen Anfangsgewicht von 20 g. Als Bestrahlungsapparatur verwendeten wir eine mit allseitiger Bleiabdeckung umgebene Spezialkonstruktion der Firma Roewag, Lützelflüh (Schweiz). Bei 15 mA und 125 kV Dosisleistung (in Luft gemessen) von 103 r/min, 3 mm Al-Filter, Abstand Fokus–Tier = 30 cm. Gruppen von je 6 Mäusen wurden in einem in 6 Einzelzellen unterteilten Plastikkästchen bestrahlt, das sich auf einer rotierenden Plattform befand (7,5 Umdrehungen/min). Die zu untersuchenden Substanzen wurden in verschiedenen Dosen und Intervallen vor und zum Teil nach der Bestrahlung parenteral verabreicht. Die allgemein verwendete Strahlendosis, welche bei 95% der Kontrolltiere innert 30 Tagen zum Exitus führte, betrug 800 r.

Ergebnisse (siehe Tabellen I und II)

Apresolin schützte in einer Dosis von 10 mg/kg, subkutan oder intraperitoneal verabreicht, mit einer LD_{95} bestrahlte Mäuse optimal, wenn es kurz vor oder auch *nach* der Bestrahlung verabreicht wurde. Fasst man zum Beispiel die 10 min vor Bestrahlung subkutan oder intraperitoneal behandelten Tiere zusammen, so überlebten von 126 Tieren 38, das heisst 30,2%; von 24 Mäusen, welche 10 min nach erfolgter Bestrahlung 10 mg/kg Apresolin s. c. erhielten, überlebten 11, das heisst 46%. Die übrigen mit 10 mg/kg erzielten Ergebnisse geben lediglich Hinweise dafür, dass Apresolin möglicherweise auch noch 3 h vor oder 30 min nach Bestrahlung schwach wirksam sein könnte. In Dosen von 3,30 oder 100 mg/kg, 10 min vor Bestrahlung verabreicht, besass Apresolin keine Schutzwirkung. Das Wirkungsoptimum von Apresolin scheint demnach in einem relativ engbegrenzten Dosenbereich zu liegen.

Tabelle I

Schutzwirkung von Apresolin bei Ganzkörperbestrahlung mit 800 r (LD_{95}) an der Maus

Dosis (mg/kg)	Applikationsart	Intervall vor (−) oder nach (+) Bestrahlung	*n*	Überlebende nach 30 Tagen
3	s. c.	−10 min	36	4
3	i. p.	−10 min	24	2
10	s. c.	−16 h	24	4
10	i. p.	−16 h	24	4
10	s. c.	− 5 h	24	3
10	i. p.	− 5 h	12	2
10	s. c.	− 3 h	24	3
10	i. p.	− 3 h	24	4
10	s. c.	−10 min	72	27
10	i. p.	−10 min	54	11
10	s. c.	+10 min	24	11
10	s. c.	+30 min	36	4
10 + 10	s. c. + s. c.	− 3 h + 3 h	24	1
30	s. c.	−10 min	24	1
30	i. p.	−10 min	24	0
100	i. p.	−10 min	24	0

Tabelle II

Schutzwirkung von C. 5864-Su bei Ganzkörperbestrahlung an der Maus

Dosis (mg/kg s. c.)	Intervall vor (−) oder nach (+) Bestrahlung	*n*	Überlebende nach 30 Tagen
0,3	−10 min	30	1
1	−24 h	48	4
1	− 5 h	48	14
1	−10 min	54	19
1 + 1	−24 h + −10 min	24	9
3	−10 min	12	0

Bei C. 5864-Su betrug die optimale Dosis etwa 1 mg/kg und war damit etwa 10mal kleiner als bei Apresolin. Zudem schützte C. 5864-Su noch bis zu 5 h vor Bestrahlung; durch zweimalige subkutane Injektion von je 1 mg/kg 24 h und 10 min vor Bestrahlung konnte die Schutzwirkung des Präparats verbessert werden. Grössenordnungsgemäss ist somit die wirksame Dosis von C. 5864-Su derjenigen des in absoluten Dosen bisher wirksamsten Schutzkörpers, Reserpin, vergleichbar, ohne allerdings dessen lange Wirkungsdauer zu erreichen.

[1] M. Langendorff *et al.*, Strahlentherapie *104*, 338 (1957).

[2] H. M. Patt *et al.*, Science *110*, 213 (1949). – H. M. Patt und P. L. Straube, Proc. Soc. exp. Biol. Med., N. Y. *84*, 702 (1953). – Z. M. Bacq und A. Herve, Bull. Acad. Roy. méd. Belg. *17*, 13 (1952).

Gemessen an der Überlebensrate stellen sich anderseits Serotonin[3] oder AET (β-Aminoäthyl-isothiuronium)[4,5] in subtoxischen Dosen besser als Apresolin oder C. 5864-Su. Bemerkenswert erscheint uns jedoch die Tatsache, dass Apresolin und C. 5864-Su ihre Schutzwirkung in Dosen entfalten, die zu keinen äusserlich erkennbaren Nebenwirkungen führen und etwa $^1/_{20}$–$^1/_{50}$ der akuten LD_{90-100} für die jeweilige Substanz darstellen. Mit AET, das bei neutralem pH eine Umlagerung in 2-Mercapto-äthylguanidin (MEG) erfährt[4,5], wurde zum Beispiel die relativ gute Schutzwirkung erst mit einer Dosis erzielt, die bei etwa der Hälfte der unbestrahlten ♀ Mäuse innert 2 Tagen zum Tod führt. Interessanterweise variiert der Schutzeffekt von AET für ein und dieselbe Dosis je nach Mausstamm sehr beträchtlich (33–88%)[5].

Von verschiedenen Autoren ist auf gewisse Analogien zwischen O_2- bzw. Peroxyd-Vergiftung und dem nach Ganzkörperbestrahlung auftretenden Syndrom hingewiesen worden[6,7]; ausserdem treten bei bestrahlten Mäusen «organische» Peroxyde in Mengen auf, die bei unbestrahlten Tieren zum Tode führen[8]. Es erschien deshalb von Interesse, Apresolin und C. 5864-Su sowie Reserpin auch auf eine eventuelle Schutzwirkung gegenüber einem organischen Peroxyd zu prüfen. Als solches wählten wir Cyclohexanonperoxyd[9]; dieses führte, mit Propylenglykol verdünnt, an unserem Tiermaterial bei 87 von insgesamt 94 Mäusen in einer Dosis von 30–40 mg/kg i.p. (je nach Charge) zu einem über 10 Tage verzettelten Absterben. Das wenig charakteristische Vergiftungssyndrom bestand aus zunehmender Apathie, Abkühlung und Gewichtsverlust; hämatologische Veränderungen wurden nicht beobachtet, dagegen wiesen die gestorbenen Mäuse höhere relative Lungengewichte mit Blutanschoppung und gelegentlicher Leberverfettung auf. Gegenüber einer solchen Peroxydvergiftung schützten nur C. 5864-Su und Reserpin, während Apresolin in der angewandten Dosierung und unter den gewählten Versuchsbedingungen keine erkennbare Schutzwirkung entfaltete. Ähnlich wie bei der Ganzkörperbestrahlung bedarf Reserpin zur Entwicklung seines Wirkungsoptimums einer Latenzzeit von einigen Stunden; ein Schutzeffekt liess sich nach einer Einzeldosis von 1 mg/kg s. c. noch nach 48 h nachweisen (siehe Tabelle III).

Die Frage nach den für die Strahlenschutzwirkung von Apresolin und C. 5864-Su verantwortlichen Faktoren lässt sich angesichts der mannigfaltigen pharmakodynamischen und biochemischen Aktivität der beiden in Frage stehenden Substanzen nicht einfach beantworten. Von den Wirkungsqualitäten, die auch von andern Autoren in Verbindung mit verschiedenen Schutzsubstanzen[10] zur Erklärung herangezogen werden, stechen im Falle von Apresolin und C. 5864-Su zwei Funktionen besonders hervor: 1. Die in unsern Laboratorien in anderem Zusammenhang nachgewiesene Chelatbildung[11,12] und eine damit zusammenhängende prooxydative oder antioxydative Wirkung auf die metallkatalysierte Oxydation biogener Amine[12,13], 2. Anhäufung, bzw. Freisetzung von biogenen Aminen. Es erscheint nicht unwahrscheinlich, dass die Strahlenschutzwirkung von Apresolin und C. 5864-Su eine Resultante beider Mechanismen darstellt.

Tabelle III

Wirkung von Apresolin, C. 5864-Su und Reserpin gegenüber Vergiftung mit Cyclohexanonperoxyd an der Maus

Behandlung (in Klammern: Dosis in mg/kg, s. c.)	Intervall vor (−) oder nach (+) Peroxyd	*n*	Überlebende nach 10 Tagen
Kontrollen	—	94	7
Apresolin (10) . . .	− 3 h	20	2
Apresolin (10) . . .	− 1 h	34	1
Apresolin (10) . . .	−10 min	12	2
C. 5864-Su (1) . . .	− 3 h	10	2
C. 5864-Su (1) . . .	− 1 h	30	16
C. 5864-Su (1) . . .	− 5 min	10	4
C. 5864-Su (1) + C. 5864-Su (1) . .	−24 h / − 1 h	20	11
Reserpin (1)	−48 h	36	9
Reserpin (1)	−24 h	46	9
Reserpin (1)	−12 h	36	8
Reserpin (1)	− 6 h	36	10
Reserpin (1)	− 3 h	10	8
Reserpin (1)	− 1 h	10	3
Reserpin (3)	− 1 h	10	6
Reserpin (1)	+ 1 h	10	2

Anderseits zeigen unsere Ergebnisse, dass gegenüber Ganzkörperbestrahlung wirksame Substanzen nicht notwendigerweise gegen Peroxyde schützen müssen, und dass eine pharmakologische Dissoziation der zwei Schutzwirkungen möglich ist.

R. Jaques und R. Meier

Forschungslaboratorien der CIBA Aktiengesellschaft, Pharmazeutische Abteilung, Basel, 4. November 1959.

Summary

Mice exposed to a lethal total-body X-irradiation were partly protected by comparatively small doses of 1-hydrazinophthalazine (Apresoline®), and even smaller ones of [2-(octahydro-1-azocinyl)-ethyl]-guanidine (C. 5864-Su, guanethidine), administered prophylactically. On the other hand, only C. 5864-Su and reserpine, but not Apresoline, afforded protection against organic peroxide intoxication.

[3] H.-J. Melching *et al.*, Naturwissenschaften *45*, 545 (1958).
[4] U. Hagen, Persönliche Mitteilung (1956).
[5] P. Urso *et al.*, Blood *13*, 665 (1958). – D. G. Doherty und W. T. Burnett, Proc. Soc. exp. Biol. Med., N. Y. *89*, 312 (1955).
[6] R. Gerschman *et al.*, Science *119*, 623 (1954); Proc. Soc. exp. Biol. Med., N. Y. *86*, 27 (1954); Proc. Soc. exp. Biol. Med., N. Y. *85*, 75 (1954).
[7] V. J. Horgan und J. St. L. Philpot, Brit. J. Radiol. [N. S.] *27*, 63 (1954); Radiobiology Symposium, Liège (Butterworth, London 1954).
[8] V. J. Horgan *et al.*, Biochem. J. *67*, 551 (1957).
[9] Hersteller: Elektrochemische Werke, München.
[10] Z. M. Bacq *et al.*, Bull. Acad. Roy. méd. Belg. *18*, 226 (1953). – H. Langendorff, R. Koch und U. Hagen, Arch. int. Pharmacodyn. *100*, 1 (1954). – U. Hagen, Arzneim.-Forsch. *6*, 384 (1956).
[11] R. Jaques, J. Tripod und R. Meier, Arch. exp. Path. Pharmak. *230*, 26 (1957).
[12] E. Tanner, Dissertation, Bern 1958.
[13] R. Meier und W. Schuler, Helv. physiol. Acta *15*, 284 (1957).

Déplacement de la radioactivité dans le ganglion cervical supérieur après injection de ^{35}S-Méthionine

Si l'accord est unanime pour reconnaître que les cellules nerveuses sont le siège d'une synthèse protéique intense, la destinée des protéines qu'elles élaborent est envisagée de façon apparemment diverse. Ces divergences semblent dues à la difficulté d'interpréter les autoradiographies du système nerveux central dont la structure complexe rend particulièrement difficile l'analyse des prolongements neuroniques[1–3]. Aussi nous sommes-nous adressés au ganglion cervical supérieur qui est un centre nerveux relativement élémentaire auquel arrivent des fibres préganglionnaires et d'où partent des fibres postganglionnaires bien individualisées[4]. Sa position anatomique, hors de la barrière sang-cerveau, et sa structure histologique simple en font un matériel favorable pour étudier les variations de radioactivité de ses constituants.

21 rats Wistar de 150 $\pm$ 10 g reçoivent une injection unique intrapéritonéale de ^{35}S-(DL)-méthionine, d'activité spécifique 19,4 mC/*mM*, à la dose de 2 μC/g de poids d'animal. 9 sont sacrifiés 30 min après l'injection, 9 autres 24 h après et les 3 derniers au bout de 72 h. Les ganglions sont prélevés, fixés au formol à 10%, lavés à l'acide trichloracétique à 5% et inclus à la paraffine. Les autoradiographies sont réalisées par coulage d'émulsion Ilford G 5 sur des coupes histologiques de 5 μ.

Les ganglions prélevés 30 min après l'injection présentent une radioactivité maximale au niveau des corps cellulaires des neurones (fig. 1); les fibres pré- et postganglionnaires ne sont le siège que de quelques trajectoires localisées autour de noyaux schwanniens. 24 h après l'injection, le maximum de radioactivité se superpose toujours aux neurones mais les fibres postganglionnaires (fig. 2) ont fortement noirci l'émulsion à l'opposé des fibres préganglionnaires dont la densité en grains d'argent est 2 à 4 fois plus faible (fig. 3). La densité moyenne en grains des fibres postganglionnaires devient 6 à 8 fois plus intense entre 30 min et 24 h, alors que la radioactivité des corps des neurones a au maximum triplé (fig. 4 et 5). En 72 h, la radioactivité décroît.

Cet accroissement du nombre de grains dans les fibres postganglionnaires est un phénomène évident mais difficile à interpréter en toute certitude. Si la répartition précoce des trajectoires, 30 min après l'injection, se fait essentiellement dans la zone des cellules schwanniennes, par contre 24 h après, on ne retrouve aucun siège de prédilection. En admettant que la radioactivité soit due aux seules cellules schwanniennes, on devrait retrouver une intensité du même ordre dans les fibres préganglionnaires, or il n'en est rien. On peut envisager que le supplément de radioactivité acquis par les fibres postganglionnaires est lié à une activité cellulaire autre que celle des cellules de Schwann; ceci semble confirmé par la décroissance progressive de la radioactivité à mesure que l'on s'éloigne de l'origine des fibres postganglionnaires. Tout en faisant la part des différences de vitesse de renouvellement mises

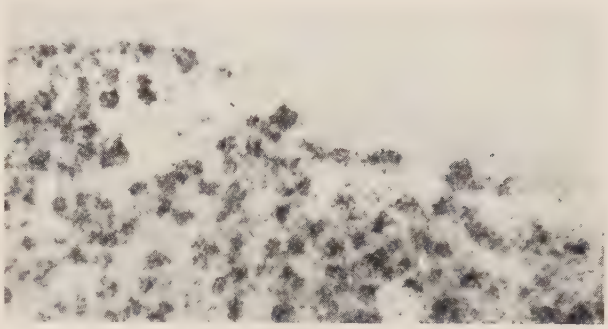

Fig. 1.—30 min après l'injection; pose: 28 jours
L'origine des fibres postganglionnaires est pauvre en grains; au contraire, les corps des neurones témoignent d'une incorporation intense de ^{35}S-Méthionine

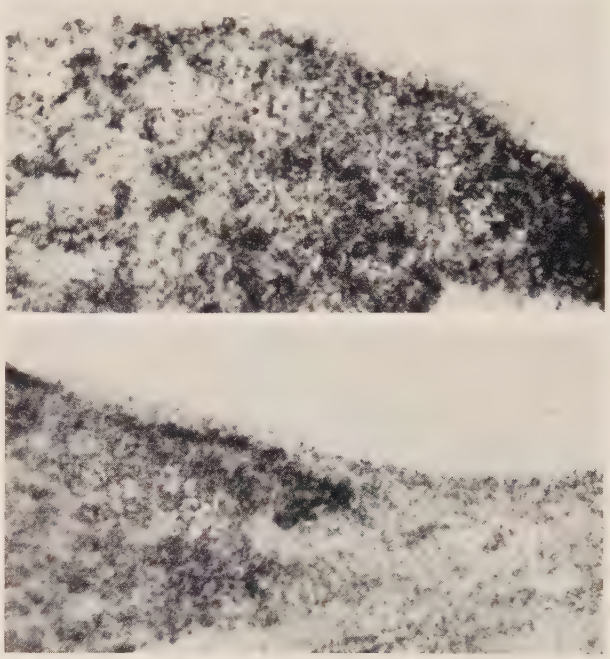

Fig. 2 et 3.—24 h après l'injection; pose: 28 jours
La figure 2 montre l'origine des fibres postganglionnaires (à droite) dont la densité en grains est presque analogue à celle des neurones. La figure 3 représente l'autre pôle du même ganglion avec l'arrivée (à droite) de fibres préganglionnaires dont la radioactivité est nettement plus réduite.

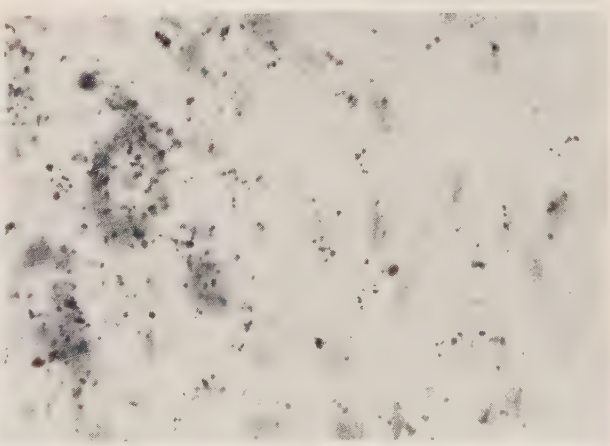

Fig. 4.—30 min après l'injection; pose: 4 jours
Les trajectoires se superposent aux corps des neurones; à droite, les grains se trouvent dans le voisinage des cellules schwanniennes dont on aperçoit les noyaux

[1] C. P. Leblond, N. B. Everett et B. Simmons, Amer. J. Anat. *101*, 225 (1957).
[2] B. Schultze et W. Oehlert, *Radioaktive Isotope in Klinik und Forschung*, vol. 3 (1958), p. 68.
[3] H. Koenig, J. biophys. biochem. Cytol. *4*, 785 (1958).
[4] B. Droz et J. Verne, 4e Congr. intern. Biochem., Vienne (1958), 6–24.

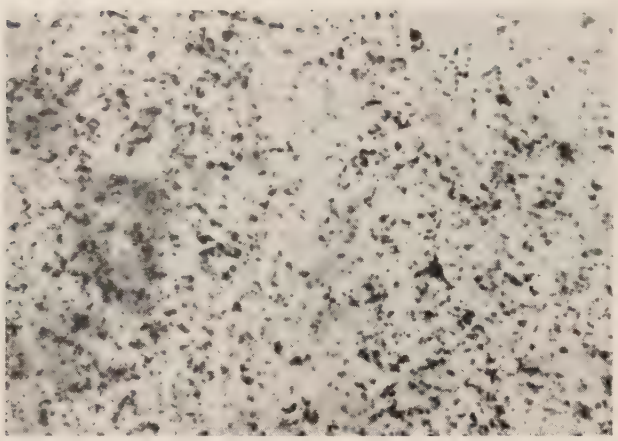

Fig. 5. — 24 h après l'injection; pose: 4 jours
La densité des grains augmente dans les neurones et davantage encore dans les fibres postganglionnaires

en évidence dans les protéines nerveuses[5,6], il reste à démontrer la réalité d'une migration des protéines élaborées par les cellules ganglionnaires[7].

J. Verne et B. Droz

Laboratoire d'histologie, Faculté de médecine de Paris, le 2 octobre 1959.

Summary

A migration of radio-activity is shown in the postganglionic nerves of the sympathetic ganglion, if an interval is allowed to elapse between the injection of Methionine ^{35}S and sacrifice. This process suggests a migration of proteins elaborated by the neurons.

[5] A. V. Palladin et N. M. Poljakowa, 4e Congr. Intern. Biochem., Vienne (1958), 2–12.
[6] H. Waelsch, 4e Congr. Intern. Biochem., Vienne (1958), Sympos. III.
[7] S. Ochs et E. Burger, Amer. J. Physiol. *194*, 499 (1958).

Contrasting Tail and other Responses to Morphine and Reserpine in Rats and Mice[1]

Introduction. The mouse tail reaction to morphine described by Straub[2] and Hermann[3] has proved of value in pharmacological studies for assessing the effects and interaction of various drugs on the central nervous system, *vide* Holten[4].

During work at present in progress we have observed in rats a tail reaction to Reserpine which seems to be the opposite to the Straub-Hermann reaction to morphine in mice, and as this latter has been so useful to pharmacologists in the past, we feel that tail reactions of these rodents to Reserpine should be described and contrasted with that seen after morphine.

[1] This work was aided by a grant from the Medical Research Council.
[2] W. Straub, Dtsch. med. Wschr. *38*, 1462 (1911).
[3] O. Hermann, Biochem. Z. *39*, 216 (1912).
[4] C. H. Holten, Acta pharmacol. toxicol. *13*, 113 (1957).

Response to morphine. Four male albino rats weighing 110–410 g injected subcutaneously with 10 mg/kg morphine sulphate, showed a tail reaction within 30 min similar to, but less pronounced than, the Straub-Hermann tail sign in mice. The normal rat carries its tail horizontally along the ground and if the tail is lifted up, it returns rapidly to the horizontal (Fig. 1). After morphine the tail tends to be slightly raised though still horizontal, but if lifted up remains for some time in an almost vertical position while the animal moves around (Fig. 2), and then slowly returns to the horizontal. That is to say, the tail is curled upwards and also lacks tone. Rats given morphine show no abnormal grasping reflex and their eyes, hair, and posture remain normal, but there is some increase in activity when the drug effect is at its maximum.

Fig. 1. — Normal rat R/1/11 weight 360 g

Fig. 2. — R/1/11 1 h after 10 mg/kg Morphine sulphate subcutaneously. Note curling up tail and open eyes

Response to Reserpine. Thirteen male albino rats weighing 130–420 g injected subcutaneously with 5 mg/kg Reserpine showed a tail reaction diametrically opposite to that seen after morphine. This appeared in some cases 3 or 4 h, but in others not for about 18 h, after injection, continued to be very marked for two days, and thereafter usually decreased. The response was quite unmistakable and recognised by a marked increase in tone in the tail, and by the fact that it was slapped down after being lifted to the vertical position and released. But the most striking phenomenon was seen when a finger was placed under the proximal portion of the tail and the tail lifted with a slow stroking movement. When this was done the tail curled down and round the finger with a grasping movement and remained flexed (Fig. 3).

Fig. 3.—R/1/12 340 g, $4^1/_2$ h after 5 mg/kg Reserpine subcutaneously. Note down curling tail and closed eyes

This reaction can be facilitated during the onset and when the effect of the drug is diminishing, if the stimulus is applied repeatedly by stroking the length of the tail with a finger or pencil placed underneath it.

Concurrent with the tail reaction the rats also developed a very strong grasp reflex in all four paws, similar to that seen in monkeys after Reserpine. This reflex could be demonstrated by placing a thick wire or small screwdriver in the fore-paws and gently raising the rat until it was suspended (Fig. 4). During this stage some rats tended to use the mouth to hang on with, and to curl up and grasp the wire with all four paws. The Reserpine monkey will also do this latter and cling on to an arm extended horizontally with all four limbs, Cole and Glees[5].

Fig. 4.—R/1/12 5 h after 5 mg/kg Reserpine subcutaneously showing strong grasp reflex

Although the duration of the tail and grasping reflexes varies in individuals, we have found that they tend to be more marked and to last longer in animals over 300 g. Other typical Reserpine phenomena observed include decrease in activity, piloerection, diarrhoea, closing of the eyes, tremor, abnormal and hunched posture.

[5] J. Cole and P. Glees, Lancet *1*, 338 (1956).

Table I sets out the contrasting morphine and Reserpine effects.

Table I

Contrasting Morphine and Reserpine effects in rats

	10 mg/kg Morphine	5 mg/kg Reserpine
Tail	Curls up	Curls down
	Weak tone	Strong tone
Grasp reflex	Normal	Very strong
Eyes	Open	Closed
Piloerection	Absent	Present
Tremor	Absent	Present
Diarrhoea	Absent	Present
Activity	Increased	Decreased
Posture	Normal	Abnormal

Also, two rats showing the full Reserpine syndrome were given 10 mg of Methyl-phenidate (Ritalin) subcutaneously. Within 20 min the Reserpine signs had almost disappeared, but they reappeared 12 h later. This confirms the antagonism between Methyl-phenidate and Reserpine reported by Cole and Glees in monkeys.

Tail responses to Morphine and Reserpine in mice. The typical Straub-Hermann tail sign was observed in two mice given 10 mg/kg morphine subcutaneously for comparison with the rats. In ten mice given 5 mg/kg Reserpine subcutaneously, although the tendency for the tails to curl round the finger when lifted up was perhaps not quite so marked as in the rats, there was pronounced increase in tone and the tail was 'pressed' down on a flat surface, and would curl down over the edge of a table.

Mice in this condition given morphine of a dose up to 20 mg/kg held their tails at an angle of about 45°, but such tails unlike the true Straub-Hermann tail tended to grasp when a finger was placed underneath them. This general observation made in mice for comparison with the tail reactions to Reserpine and morphine seen in our rats might indicate, as Schneider[6] suggets, that the Reserpine-morphine relationship is competitive at various levels of the central nervous system.

The careful study of Tripod *et al.*[7], in which they found that in mice Reserpine antagonised the morphine tail-sign but was synergistic with morphine on the pupil reaction, supports the view that these two drugs are competitive at various levels rather than directly antagonistic as Reserpine and Methyl-phenidate appears to be. In fact these authors state 'that a true antagonism is unlikely'.

Controls. We tested the tail and grasp reflexes of 50 normal albino rats of both sexes and varying weight from 25–580 g. The results are shown in Table II.

Both the grasp and tail signs found were little more than equivocal and in no case as pronounced as those seen after the drugs. The downward curling tails which needed much facilitation, had not as much tone, nor were they slapped down as was the case after Reserpine. The two up-curling tails were also doubtful; in fact can best be described as showing a tendency to resemble the morphine tail.

[6] J. A. Schneider, Proc. Soc. exp. Biol. Med. *87*, 614 (1954).

[7] J. Tripod, H. J. Bein, and R. Meier, Arch. int. Pharmacodyn. *96*, 406 (1954).

Table II

Incidence of tail and grasp reflexes in 50 normal rats resembling those seen after Morphine or Reserpine

Weight in g	Sex	No.	Tail		Grasp reflex
			down	up	
25–54	M	6	—	—	4
25–54	F	2	—	—	—
55–99	M	3	—	—	—
55–99	F	5	—	—	—
100–249	M	10	—	1	—
100–249	F	4	—	1	1
250–580	M	15	4	—	—
250–580	F	5	—	—	—

We are grateful to CIBA Laboratories for supplying Reserpine.

J. Cole and D. P. Dearnaley

University Laboratory of Physiology and University Department of Biochemistry, Oxford, October 9, 1959.

Zusammenfassung

Charakteristisch verschiedene Schwanzstellungen zeigen Albino-Ratten und -Mäuse als Morphium- bzw. Reserpinreaktion. Verstärkte Schwanzreaktionen erhält man bei der Maus nach Morphium, bei der Ratte nach Reserpin. Methylphenidat erweist sich bei der Ratte antagonistisch gegen Reserpin, während im Mäuseversuch nach Reserpin injiziertes Morphium kompetitiv zu wirken scheint.

DISPUTANDUM

Lignin and the Formation of Wood

The paper by F. F. Nord and W. J. Schubert on the title subject in Experientia *15*, 245 (1959) requires several amendments and supplementary comments. For the sake of brevity, the discussion here has been restricted to the grossest inadequacies and discrepancies.

The best preparation of wood lignin available at present is Björkman's milled wood lignin (MWL)[1]. MWL prepared from spruce wood contains 0·3 phenolic hydroxyl groups per C_6–C_3 unit, whereas the minute fraction of ligninlike material which is extractable from sawdust according to F. Brauns contains twice this amount of phenolic hydroxyl[2,3a]. Here the latter preparation was extracted from wood using acetone in preference to alcohol and then separated from lignans and other admixtures by countercurrent distribution. Nord and Schubert claim that 'enzymically liberated lignin' is identical with Brauns' 'native lignin'. This implies therefore that it is not identical with the main part of *true* wood lignin or protolignin. Thus, for instance, for Scots pine, values of 815 and 695 were found by Nord *et al.*[4] for the molecular weights of native and enzymically liberated lignin respectively. Spruce MWL on the other hand, although probably partially degraded by milling, has a molecular weight of 11,000[1], in the same range as that of Freudenberg's biosynthetic lignin[3b].

Brown and Neish[5] were the first to show that radioactive shikimic acid introduced into plants is incorporated into lignin and that radioactive vanillin etc. is formed on oxidation of such lignin. Independently, Eberhardt and Nord[6a] confirmed this in more elaborate experiments. The role of shikimic acid in lignin formation is just the same as in all other cases of biosynthesis of C_6–C_3 substances. Various American authors have shown that shikimic acid is transformed into prephenic acid and thence into phenylpyruvic acid or cinnamic acid or phenylalanine. As Freudenberg has shown, labelled phenylalanine is transformed by spruce saplings either in the needles or in the cambium or en route from the needles to the cambium into coniferin, which was isolated in radioactive form from cambial sap[7].

In conifers coniferin and in other plants corresponding glucosides in addition assume the cardinal position in lignin formation. These are the immediate starting materials for lignification, as has been demonstrated by Freudenberg *et al.*[8] in the case of spruce lignin using radioactive coniferin.

Nord, Schubert and Acerbo[9] have shown that radioactive *p*-hydroxyphenylpyruvic acid is incorporated into sugar cane lignin and that such lignin forms radioactive aldehydes on oxidative degradation. This important observation does not indicate however that *p*-hydroxyphenylpyruvic acid is the only or even an essential starting material for lignin formation, as Nord and Schubert suggest. In an analogous experiment carried out with labelled ferulic acid, Freudenberg[10] found that lignin is formed which yields radioactive ketones on ethanolysis according to Hibbert. The correct interpretation of this data is that phenylpyruvic acid or cinnamic acid or phenylalanine is oxidized on the way leading to coniferin in the 4-position and in the 3-position, which is also methylated. The acids are reduced at the carboxyl group, are converted into unsaturated compounds and finally glucosidized. The absolute sequence of these operations has not yet been definitely established[11]; *p*-hydroxyphenylpyruvic acid and ferulic acid may be essential intermediates in these transformations or may merely be transformed independently in side reactions into coniferin and other glucosides. The role of *p*-hydroxyphenylpyruvic acid is rather uncertain, as it has been shown that its incorporation into conifer lignin proceeds rather unsatisfactorily[11,12].

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[3] a) K. Freudenberg, in *Biochemistry of Wood*, Proc. 4th Int. Congr. Biochem., Vol. 2, p. 125 (1959). – b) K. Freudenberg and K. Dall, Angew. Chem. *42*, 606 (1955).

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[10] K. Freudenberg, Angew. Chemie *68*, 92, 511 (1956). – S. A. Brown and A. C. Neish, Canad. J. Biochem. Physiol. *34*, 769 (1956).

[11] S. A. Brown, D. Wright, and A. C. Neish, Canad. J. Biochem. Physiol. *37*, 25 (1959).

[12] G. Billek, in *Biochemistry of Wood*, 4th Int. Congr. Biochem., Vol. 2, p. 211 (1958).

The following statements of NORD and SCHUBERT summarizing 'the process of lignification' are therefore not in clear concordance with the facts of current lignin chemistry: 'This compound (i. e. shikimic acid) is then aromatized into compounds of the type of *p*-hydroxyphenylpyruvic acid. These latter compounds then become the first, or 'primary' lignin building stones'. It ought at least to have been stated that the primary building stones are actually *p*-hydroxycinnamic alcohols.

Furthermore NORD and SCHUBERT give the impression that nothing is known about the oligomeric intermediates of lignin formation. In this respect, attention is called to work done in Heidelberg in recent years in which so far ten different intermediates have been isolated. Inexplicably, they have also neglected to mention the well established role of the glucosides, their hydrolysis prior to lignification by a β-glucosidase present between cambium and wood in trees and the dehydrogenative condensation of the free phenolic aglycons brought about by oxidizing enzymes at the site of lignification in the plant.

All in all, NORD and SCHUBERT have merely described a pair of random steps isolated from the complexity of the total process of lignification and hence convey an oversimplified, distorted picture of the overall scheme. For a condensed but more complete survey of the biosynthesis and constitution of lignin, a recent article by FREUDENBERG[13], is recommended.

J. M. HARKIN

Forschungsinstitut für die Chemie des Holzes und der Polysaccharide, Chemisches Institut der Universität Heidelberg, 5. August 1959.

A Rebuttal to the Disputandum of J. M. Harkin

The Disputandum by HARKIN to the paper of NORD and SCHUBERT in this Journal[14] is characteristic of the style of the Heidelberg school, but is unconvincing.

The statement is made that 'The best preparation of wood lignin available at present is A. BJÖRKMAN's milled wood lignin (MWL)'. But until the structure of natural lignin has been proved, one cannot say what is the 'best' preparation of lignin, for until such time as its structure becomes known, one cannot know what the *exact* properties of natural lignin will be, and so one cannot know how closely his own preparation approaches lignin *in situ*. While we do not disparage the value and importance of BJÖRKMAN's preparation, we and indeed others feel that it is questionable to call it, or any other preparation, 'best' at this time.

We do indeed claim that enzymatically liberated lignin is identical with BRAUNS' native lignin, but this certainly carries no implication that it is not identical with the main part of true wood or bagasse lignin. Once again, the author has arbitrarily selected Spruce MWL as a standard of reference, and then he totally confounds the issue by reference to FREUDENBERG's 'biosynthetic lignin', which of course, is not a natural, wood-produced lignin at all, but an artifact[15].

The statement is made 'Brown and Neish were the first to show that radioactive shikimic acid introduced into plants is incorporated into lignin and that radioactive vanillin, etc. is formed on oxidation of such lignin. Independently, EBERHARDT and NORD confirmed this in more elaborate experiments'. To keep the record straight, let it be known that BROWN and NEISH's letter was published in the April 16, 1955 issue of Nature, while the Fordham letter was published in Arch. Biochem. Biophys. in the April, 1955 issue.

Furthermore, no attempt is made to deny that 'The role of shikimic acid in lignin formation is just the same as in all other cases of biosynthesis of C_6–C_3 substances', or that 'Various American authors have shown that shikimic acid is transformed into prephenic acid and thence into phenylpyruvic acid or cinnamic acid or phenyl-alanine'. We simply claim to have called attention (see above), to the intervention of shikimic acid in lignification, as compared with other biosyntheses of aromatic compounds, a possibility which was earlier completely overlooked by FREUDENBERG[16].

The statement is made that coniferin and other glucosides are the 'immediate starting materials for lignification'. But why this arbitrary selection of the glycosides as the starting point of the process? We feel that the origin of the glycosides themselves is just as integral a part of the *total* process of lignification as the eventual conversion of these compounds into lignin.

Regarding *p*-hydroxyphenylpyruvic acid, it is claimed that its role is 'rather uncertain as it has been shown that its incorporation into conifer lignin proceeds rather unsatisfactorily'. But NEISH[17] states that '*p*-hydroxyphenylpyruvic acid-3-C^{14} can be converted to guaiacyl or syringyl lignin in wheat but not in buckwheat (*Fagopyrum tataricum*) or sage (*Salvia splendens*)'. This difference in biosynthetic abilities among different species was later interpreted[11] as follows: 'Neither *p*-hydroxyphenylpyruvic acid nor *p*-hydroxyphenyl lactic acid is a general intermediate in lignification, and differences noted here and in previous papers between grasses and nongrasses probably result from the unique ability of grasses to convert *p*-hydroxyphenyl lactic acid to *p*-hydroxy cinnamic acid'. Since the sugar cane plants employed in our experiments are considered to be 'grasses', they are thus able to utilize *p*-hydroxyphenylpyruvic acid for lignin biosynthesis, as has been demonstrated in this laboratory[15].

The statement is made that 'It ought at least to have been stated that the primary building stones are actually *p*-hydroxycinnamic alcohols'. But, if this was not explicitly stated, it was certainly clearly implied. Thus, on p. 252 of the paper in this Journal, it was stated that 'The building stone of lignin possesses a phenylpropane carbon structure (Figure 4b). Lignin building stones, although having the same basic phenylpropane carbon structure, may be of at least three types, the vanillyl (Figure 12a), syringyl (Figure 12b), or *p*-hydroxyphenylmethyl (Figure 12c) structure'. Hence, obviously the reader who superimposed the phenylpropane carbon structure of Figure 4b upon the vanillyl structure of Figure 12a would have deduced the structure of coniferyl alcohol, which in fact was presented in Figure 2 on page 246.

[13] K. FREUDENBERG, Nature *183*, 1152 (1959).

[14] F. F. NORD and W. J. SCHUBERT, Exper. *15*, 245 (1959).

[15] F. F. NORD, W. J. SCHUBERT, and S. N. ACERBO, Naturwissenschaften *44*, 35 (1957).

[16] K. FREUDENBERG *et al.*, Liebigs Ann. *575*, 145 (1952); *584*, 54 (1953).

[17] A. C. NEISH, in the Discussion of the paper of G. BILLEK in *Biochemistry of Wood*, Vol. 2 of the Proceedings of the Fourth International Congress of Biochemistry, p. 213 (London 1959).

Relative to the oligomeric intermediates in lignin formation, comments on them were made on p. 253 of the original paper. No reference was made to the 'intermediates' isolated at the Heidelberg school since the authors wished to restrict themselves to investigations of the processes occurring in woody plants, whereas the 'intermediates' referred to are the result of the *in vitro* action of an ill-defined enzyme system from mushrooms, which results may in fact have no relation at all to the process of lignification.

Accordingly, the concluding statement of the Heidelberg authors' regarding the Fordham picture of the overall scheme of lignification (as also reviewed by Evans[18]) may be dismissed as a purely subjective analysis.

Carmine J. Coscia

Department of Organic Chemistry and Enzymology, Fordham University, New York, September 24, 1959.

In reply to the rebuttal by Coscia of my objections to the paper by Nord and Schubert[14], I should like to clarify the following points.

I repeat that Björkman's milled wood lignin (MWL) is the best lignin preparation *available at present*, since it can be obtained repeatedly in yields of over 50%[1] and is likely to be more representative of natural lignin *in situ* than Brauns soluble lignin, the yield of which represents only 2–3% of the total lignin. If enzymically liberated lignin is identical with Brauns lignin, which is not identical with MWL, then it cannot be identical with the main part of wood lignin. The conformity of Brauns lignin and enzymically liberated lignin is also open to question because of the low methoxyl content of the latter[19]. Freudenberg's biosynthetic lignin is chemically identical with Björkman lignin but not with Brauns lignin.

It is for the sake of simplicity in experimentation that spruce lignin, which contains practically only guaiacylpropane residues, is preferred by us as a reference material for lignin studies.

Relative to the discovery that shikimic acid is a precursor of lignin, my comments are in accordance with statements by Kratzl[20]. Furthermore, the letter by Eberhardt and Nord 'on the phase sequence of methyl *p*-methoxycinnamate and its possible relation to lignification'[6a] contains no report of shikimic acid's being an intermediate in the formation of lignin. It states that 'the formation of shikimic acid is presumed' in the formation of methyl *p*-methoxycinnamate – which in turn *may* be related to lignification – and in a reaction flow sheet, shikimic acid appears in brackets with a question mark, but is otherwise unmentioned. Nord's *practical* work relating shikimic acid to lignin only appeared in 1956[6b], whereas Neish's 1955 publication was complete with experimental data. The papers by Freudenberg[16] cited by Coscia, although dealing with shikimic acid, describe studies relative to its stereochemical configuration and do not have the remotest connection with lignin chemistry.

The glucosides were not designated as the ultimate starting point of the *total* process of lignification – which is in fact atmospheric CO_2 – but as the 'immediate starting materials for lignification'; they are the culminating substances of a large number of phenylpropane derivatives which are all potential precursors of lignin. They are, however, the only precursors to have been so far isolated from plants and are present in much higher stationary concentration than any of the others. The transformation of phenylalanine into coniferin by spruce saplings[7] illustrates the important central position of coniferin in lignification. The eventual conversion of the *p*-hydroxycinnamic alcohols into lignin is lignification in the strict sense; the conversion of, for example, shikimic acid into *p*-hydroxyphenylpyruvic acid is not; this latter process occurs in many organisms (e.g. *Lentinus lepideus*) which are by no means lignified.

The inability of plants other than Gramineae to convert *p*-hydroxyphenylpyruvic acid into lignin is the uncertainty implied in my original statement; it can only be metabolized if converted into *p*-hydroxycinnamic acid in the plant[11]. We have restricted ourselves for reasons mentioned above to conifer lignin, in which this transformation does not occur. It is indeed some progress that Nord *et al.* now consider that the *p*-hydroxycinnamic alcohols are intermediates of lignin formation.

The references to 'the preliminary stage of lignification' at which 'dimerization or trimerization has already occurred' convey no picture of what the oligomeric intermediates of lignin are like or how they have been formed. The dehydrogenative polymerization of coniferyl alcohol reported by Freudenberg *et al.* is an attempt at an *in vitro* reproduction of the *in vivo* process occurring in woody plants and uses the same enzymes as occur there. We call the first (dimeric) products of this dehydrogenation intermediates of lignin formation or secondary building stones. Careful investigations suggest that these intermediates are conclusive for conifer lignin in nature.

The enzyme – laccase – used to produce biosynthetic lignin can no longer be termed 'ill-defined'[21], and is of widespread occurrence in plants. More recent work uses H_2O_2 and crystalline peroxidase, a system exactly equivalent to that of laccase with oxygen.

The article by Evans[18] is a review of the metabolism of aromatic compounds by lower plants, not of the overall scheme of lignification.

The *experimental* work done by Nord *et al.* is not at variance with our conception of the biogenesis and constitution of lignin, but it only partially covers the problems at issue. It is mainly the *interpretation* which Nord *et al.* give to their results that we cannot accept.

Professor Freudenberg stresses that he agrees with my first and this second Disputandum. I wish to call attention to his recent survey[22].

J. M. Harkin

Forschungsinstitut für die Chemie des Holzes und der Polysaccharide, Chemisches Institut der Universität Heidelberg, 19. Oktober 1959.

[18] C. Evans in *Encyclopedia of Plant Physiology*, vol. 10 (Springer Verlag, Heidelberg 1958), p. 498.

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[22] K. Freudenberg, Chem. Ber. *92*, LXXXIX (1959).

COGITATIONES

The Specificity of Reactions of Polycyclic Aromatics
II. Polarographic Reductions[1]

By F. T. Wallenberger*

Polarographic reductions of polycyclic aromatic hydrocarbons have been studied extensively by Wawzonek[2,3] and by Bergman[4]. Wawzonek was primarily interested in the mechanism of the reduction of conjugated and aromatic systems at the dropping mercury electrode and as related[3] polarography to addition of alkali metals to polycyclic aromatics. Bergman[4] has reported half-wave potentials ($E_{1/2}$) of 75 polycyclic aromatic hydrocarbons.

In the quest for a theoretical assessment of his extensive experimental study he has attempted to correlate graphically the number of π-electrons per molecule and the wave lengths of para-bands[5] in the UV spectra with the half-wave potentials.

While Wawzonek's mechanism studies and Bergman's experimental contributions are of extreme significance to the chemistry of polycyclic aromatics, it appears that Bergman's theoretical correlation is of limited validity and open to criticism.

The para-bands (also known as *p*-, group II-, primary, and L_a-bands)[6] as classified and defined by Clar[4,5] have been attributed to meso-C-atoms or, in other words, to excitations of para positions of these molecules in the UV spectra. Assuming this being so, Bergman's correlations should not have included data derived from 1,2-(bond) reactions (phenanthene, chrysene, etc.) but only compounds which are clearly reduced by 1,4-(conjugate) addition. There is furthermore also a certain ambiguity about the band assignments themselves.

Thus it appears that a general and comparative correlation of polarographic reductions of polycyclic aromatics has to be attempted on a different basis.

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[1] F. T. Wallenberger, Tetrahedron Letters *9*, 5–9 (1959).

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[4] I. Bergman, Trans. Faraday Soc. *50*, 829 (1954).

[5] E. Clar, *Aromatische Kohlenwasserstoffe*, 2nd Edit. (Springer-Verlag, Berlin 1953).

[6] G. M. Badger, *The Structure and Reactions of the Aromatic Compounds* (Cambridge University Press, Cambridge 1954), p. 386.

Table I
Specificity of Polarographic Reductions of Polycyclic Aromatics

Hydrocarbon in C. A. numbering and nomenclature	Reduction[a] Potential $E_{1/2}$		Diffusion[b] Current i_d		Resonance[c] Energy		Positions of Preferred Reduction
	1st	2nd	1st	2nd	Positions	kcal.	
Naphthalene	1·98		5·5		1,2	20·0	1,2; 1,4[2–4,e]
					1,4	27·6	
Anthracene	1·46		3·3		1,2	20·0	9,10[3,4]
					1,4	25·6	
					9,10	15·6	
Naphthacene	1·135	2·02	1·51	1·47	1,2	19·5	5,12[4]
					1,4	24·6	
					5,12	13·6	
Pentacene	0·86	1·92	0·053	0·094	1,2	19·4	6,13[4]
					1,4	24·6	
					5,14	13·6	
					6,13	11·6	
Phenanthrene	1·935	2·125	3·4	3·9	1,2	23·9	9,10[2–4]
					3,4	24·4	
					1,4	29·6	
					9,10	16·2	
Chrysene	1·805	2·165	5·5	2·9	1,2	23·6	5,6[2,4]
					3,4	23·3	
					5,6	18·1	
					1,4	28·6	
Dibenz(a, h)anthracene	1·545	1·97	1·85	4·3	5,6	15·7	5,6[4]
					7,12	22·6	
Pyrene	1·61	1·70 (1·80)[d]	3·25	3·3	4,5	16·0	4,5[3,4]
Benz(a)pyrene	1·36	1·59 (1·89)[d]	2·15 (1.45)[d]	2·35	1,2	17·6	6,7[3,4,f]
					6,7	14·8	
Triphenylene	1·97		8·84		1,2	25·8	1,2[4]
					2,3	32·1	
					1,4	30·0	

[a] From reference [4]; [b] from reference [4] ($i_d = I_d/\mathrm{cm}^{2/3}\, t^{1/6}$); [c] obtained or calculated from data contained in references [8,9], and [10]; [d] third wave; [e] the 1,2-dihydro T. S. is theoretically favored; [f] the possibility of 1,6-additions in polarographic reductions is under consideration.

In pursuit of an earlier suggestion of MACCOLL[7] involving relationships with the coefficient of the molecular orbital resonance energy integral, it will now be attempted to correlate the specificity and ease of polarographic reductions of polycyclic aromatics with theoretical resonance energy decreases accompanying the reaction.

Having found that the reactions proceed by 1,2-(bond) and 1,4-(conjugate) addition, it appears that the preferred type of reaction, like in ozonizations of polycyclic aromatics[1], depends upon the relative stability of various possible ortho and para dihydroaromatic intermediates and products. These relative stabilities can be assessed from resonance energies which have been calculated[8] for ortho positions of a large number of polycyclics and which are easily computed for para positions of these compounds from para-localization energies[9] according to a suggestion of WHELAND[10].

Table I comprises such a correlation of resonance energies, first half-wave potentials, and preferred course of polarographic reduction of a representative number of individual hydrocarbons.

In general it can be seen that reaction occurs preferentially at positions of lowest resonance energy values. References indicate wherever products have been studied experimentally[2] or where the significance of first and subsequent waves has been rigorously assessed in terms of first reaction step and further reduction of the primary reaction products[3].

The course of the preferred polarographic reduction with naphthalene is not entirely clear. Alkali metals are added predominantly by 1,4-addition, and electrolytic reduction in the presence of CO_2 gave reportedly both 1,2- and 1,4-dicarboxylic acid; only the latter was definitely isolated[2]. It seems that electronic repulsion and steric substituent effects favor 1,4-addition despite the more stable 1,2-dihydro transition state. This assumption is substantiated by the ease of rearrangement of 1,4- into 1,2-dihydronaphthalene[11].

In more complex polycyclic aromatic hydrocarbons (e. g. benz(a)pyrene, perylene, etc.) it was found that 1,6-oxidations occur readily with various oxidants[5] as well as with oxygen, catalyzed by 2–4% ozone[12]. This suggests that the 1,6-quinoid (dihydro) structures of these compounds are relatively more stable under given conditions than their 1,4- and 1,2-quinoid (dihydro) structures. Correspondingly one might also expect 1,6-reactions from polarographic reductions. The resonance energies (if calculated to apply to such conditions) then likewise are lower than the resonance energy decreases calculated for the most favored 1,4- and 1,2-positions.

The presented correlation for the first time explains why polarographic reductions proceed predominantly by 1,2-(bond) addition with some polycyclic aromatics and by 1,4-(conjugate) addition with others. The resonance energy correlation can be extended to compounds[4,8–10] not listed in this communications.

Furthermore, it is now also possible to assess the order of relative reactivity towards reduction (Table II) of the polycyclic aromatics listed in Table I, with pentacene as most reactive and triphenylene as least reactive. There is a linear relationship for para-reductions while the relationship for ortho-reductions rapidly levels off to a constant value 2·00 ± 20.

Table II

Reactivity of Polycyclic Aromatic towards Polarographic Reduction

Hydrocarbon in C. A. numbering and nomenclature	Reactive Positions		Resonance [a] Energy		Reduction [b] Potential	
	para	ortho	para	ortho	para	ortho
Pentacene . .	6,13		11·6		0·86	
Naphthacene .	5,12		13·6		1·135	
Benz(a)pyrene .		6,7 [c]		14·8		1·36
Anthracene . .	9,10		15·6		1·46	
Dibenz(a,h) anthracene .		5,6		15·7		1·545
Pyrene		4,5		16·0		1·61
Phenanthrene .		9,10		16·2		1·935
Chrysene . . .		5,6		18·6		2·165
Naphthalene .		1,2 [d]		20·0		1·98
Triphenylene .		1,2		25·8		1·97

[a] Obtained or calculated from data contained in references [8,9] and [10]. – [b] From reference[4]; [c] see footnote b in Table I; [d] the 1,2-dihydro T. S. is theoretically favored.

Thus it has been shown that it is possible to predict the specificity of polarographic reductions and to account for the relative reactivity of polycyclic aromatics towards reactions at the dropping mercury electrode.

Zusammenfassung

Die polarographische Reduktion polyzyklischer aromatischer Verbindungen kann als 1,2-, 1,4- oder 1,6-Addition erfolgen. Der Verlauf der Reaktionen lässt sich aus der Abnahme der Resonanzenergie ableiten.

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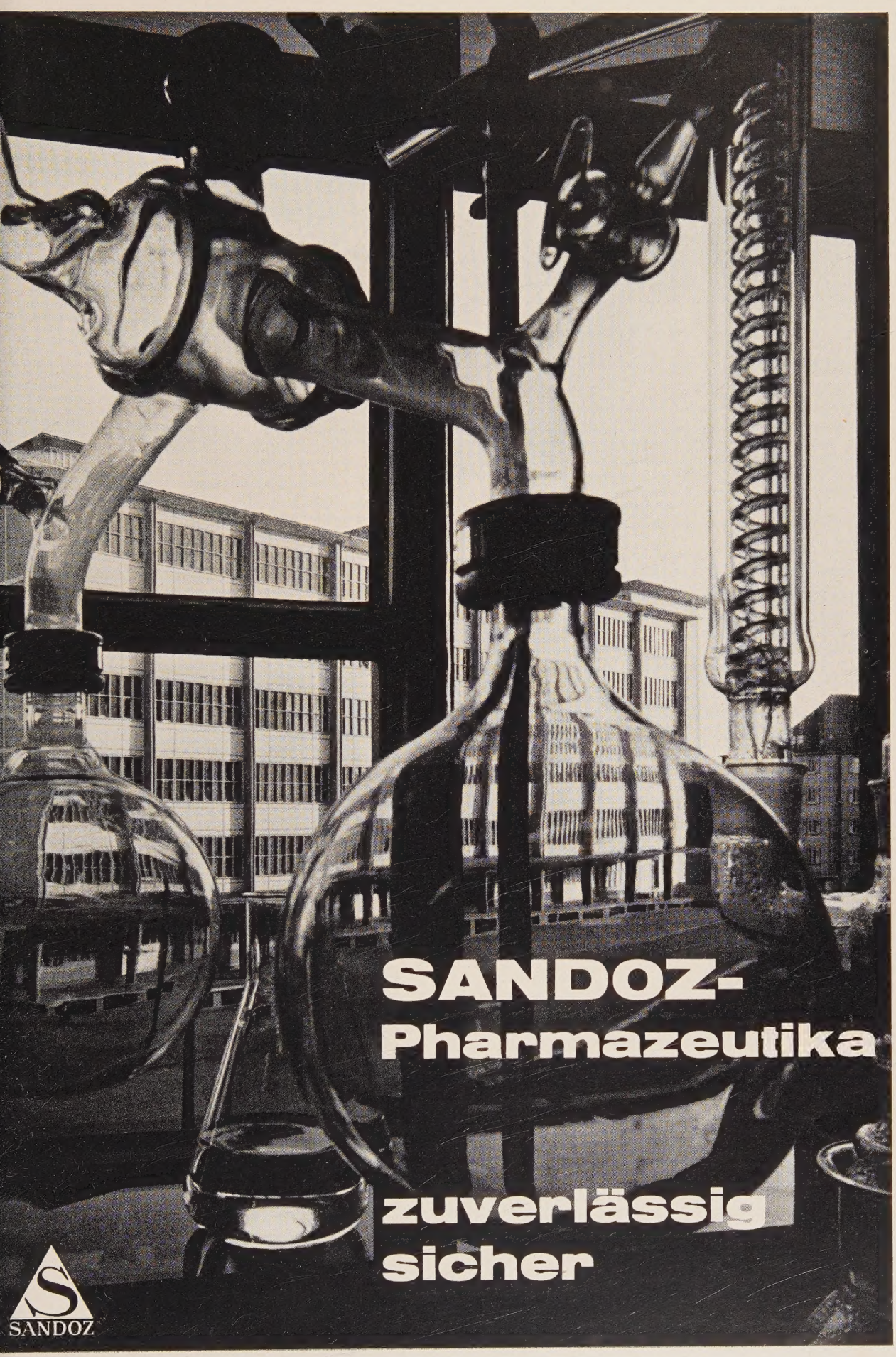
[12] E. J. MORICONI, W. F. O'CONNOR, L. B. TARANKO, B. RAKOCZY, and B. P. FÜRER, Abstracts of Papers, 136th Meeting, American Chemical Society, Atlantic City, N. J., September 1959, page 88 P.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